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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/855,682	05/16/2001	Ning Li	031855.0007	1553

26118 7590 02/28/2003

BROBECK, PHLEGER & HARRISON, LLP
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EXAMINER
EINSMANN, JULIET CAROLINE

ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 02/28/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/855,682	LI ET AL.	
	Examiner	Art Unit	
	Juliet C Einsmann	1634	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *05 December 2002* .

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-10* is/are pending in the application.

4a) Of the above claim(s) *7-9* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-6 and 10* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

1. This action is written in response to applicant's correspondence submitted 12/5/02, paper number 18. Claims 1 and 2 have been amended and claims 3-10 have been added. Claims 1-10 are pending, and are subject to restriction as set forth herein. Applicant's amendments and arguments have been thoroughly reviewed, but are not persuasive for the reasons that follow. Any rejections not reiterated in this action have been withdrawn. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action. **This action is FINAL.**

Priority

2. Applicant's amendment to the first line of the specification indicating a claim to priority to parent application 09/239796 is noted. In light of this amendment, and in light of the amendments to the claims, new grounds of rejection are set forth herein.

Sequence Rules

3. The application is in compliance with the sequence rules.

Election/Restrictions

4. Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-6 and 10, drawn to methods for screening pigs to determine those more likely to produce larger litters, classified in class 435, subclass 6.

II. Claims 7-9, drawn to kits comprising reagents which identify a genetic polymorphism, classified in class 536, subclass 24.3.

The inventions are distinct, each from the other because of the following reasons:

5. Inventions I and II are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product (MPEP § 806.05(h)). In the instant case the kits of invention II can be used in a variety of methods including nucleic acid amplification, the study of other aspects of porcine reproductive physiology, aptamer methods, and nucleic acid purification methods.

6. Because these inventions are distinct for the reasons given above and have acquired a separate status in the art as demonstrated by their different classification and recognized divergent subject matter and because inventions I-II require different searches that are not coextensive, examination of these claims would pose a serious burden on the examiner and therefore restriction for examination purposes as indicated is proper.

7. Newly submitted claims 7-9 are directed to an invention that is independent or distinct from the invention originally claimed for the stated reasons.

Since applicant has received an action on the merits for the originally presented invention, this invention has been constructively elected by original presentation for prosecution on the merits. Accordingly, claims 7-9 are withdrawn from consideration as being directed to a non-elected invention. See 37 CFR 1.142(b) and MPEP § 821.03.

Claim Rejections - 35 USC § 112

8. The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

9. Claims 1-6 and 10 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 1-2 are indefinite over the recitation "at least one marker linked to the FSH β-subunit allele(s) of SEQ ID NO: 1" because it is not clear what applicant intends to be referring to as an allele of SEQ ID NO: 1. The specification disclosed that SEQ ID NO: 1 is a retroposon whose presence within the gene encoding FSH β-subunit in a pig is an associated with smaller litter size. Thus, one allele of the FSH β-subunit gene is when SEQ ID NO: 1 is present and one alleles of the FSH β-subunit gene is when SEQ ID NO: 1 is absent. It is not clear what applicant intends when referring to alleles of SEQ ID NO: 1 itself, because this language appears to refer to variability within SEQ ID NO: 1, but in light of the specification, it is not clear that applicant has ever contemplated such variability. Thus, the claims are indefinite because the phrase "the FSH β-subunit allele(s) of SEQ ID NO: 1" lacks proper antecedent basis in the claims and the specification because neither previously refer to alleles of SEQ ID NO: 1.

Claim 10 is indefinite because it depends from non-elected claim 9, and thus the metes and bounds of claim 10 are unclear because the claim is effectively incomplete.

Claim Rejections - 35 USC § 112

10. Claims 1-6 and 10 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for methods of screening to detect the specific insertion of a retroposin comprising SEQ ID NO: 1 in the FSH β-subunit gene, does not reasonably provide

enablement for detection of any additional alleles of the FSH β-subunit. Furthermore, the specification and prior art, while being enabling for the detection of the ESR gene and the OPN gene as genes that are associated with pig litter size do not provide enablement for the detection of additional genes associated with pig litter size. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

The following factors have been considered in formulating this rejection (*In re Wands,* 858F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988): the breadth of the claims, the predictability or unpredictability of the art, the amount of direction or guidance presented, the presence or absence of working examples of the invention and the quantity of experimentation necessary.

The invention relates to methods for screening pigs to determine those more likely to produce larger litters and/or those less likely to produce larger litters. The method steps of claims 1 and 2 require determining the presence of at least one marker linked to the FSH β-subunit allele(s) of SEQ ID NO: 1. Thus, the scope of claims 1 and 2 includes using any marker linked to instant SEQ ID NO: 1 as an indicator that a pig is more or less likely to produce a larger litter. The method steps of claims 3-6 and 10 require determining the presence of at least one FSH β-subunit allele associated with pig litter size, and the determining the presence of one other gene associated with pig litter size. Claims 5 and 6 limit the scope of the "other gene" to the ESR gene or the OPN gene. The scope of claims 3-6 and 10 includes using any marker within the FSH β-subunit gene as an indicator that a pig is more or less likely to produce a larger

litter, and further, claims 3-4 and 10 encompass the detection of any possible second gene that is associated with pig litter size.

The specification contains a single working example, demonstrating that a specific inserted retroposon allele is linked was linked to litter size. Namely, the specification demonstrates that when SEQ ID NO: 1 is present in the FSH β-subunit gene of a female pig, that pig is more likely to produce a smaller litter than when SEQ ID NO: 1 is absent in the FSH β-subunit gene of the pig (See Examples 1 and 2). The specification further speculates that it is also possible to establish linkage between specific alleles of "alternative DNA markers" and the FSH β-subunit allele disclosed in the instant specification. There is not specific teaching in the specification regarding these alternative markers, besides the speculation that they may exist.

With regard to the "other genes" recited in claims 3-6 and 10, the specification teaches that the prior art provides disclosure that the OPN and ESR genes have previously been shown to be associated with pig litter size. The specification does not disclose or suggest any additional genes that might be associated with pig litter size.

The prior art includes the disclosure of an eleven base pair deletion from the FSH β-subunit which is present only in Chinese Meishan pigs (Li *et al.* Biology of Reproduction, August 1997, Volume 56, Supp. p. 119, abstract #148). (Meishan pigs are known for their prolificacy.) The prior art also discloses one other RFLP which is the result of a point mutation, but no association between this mutation and pig litter size (Rohrer *et al.* Mammalian Genome, 5, 315-317 (1994)). Thus, the prior art includes the teaching of two other possible genetic variants in the FSH β-subunit, but there is not other teaching of FSH β-subunit variants in the

prior art, nor is there clear evidence that any polymorphism found within the FSH β-subunit gene or any other "linked" gene would necessarily be linked to increased litter size. With regard to the "other" gene recited in claims 3-6 and 10, teaches a single polymorphism present in the OPN gene that is associated with pig litter size (WO 96/41892) and a single polymorphism present in the ESR gene that is associated with pig litter size (WO 92/18651).

In order to practice the claimed method commensurate with its full scope, a practitioner would be required to identify additional polymorphisms within and linked to the FSH β-subunit gene that are predictive of litter size in pigs. Further, a practitioner would be required to identify additional genes that are associated with pig litter size. The art is highly unpredictable with regard to the presence and functionality of polymorphic sites in genomic DNA. The amount of direction or guidance presented in the specification and the prior art of only three different FSH β-subunit polymorphisms compared to any pig FSH β-subunit polymorphic sequence is minimal, given that just the redundancy of the genetic code of the approximately 100 amino acid protein would allow for several thousand different sequences while when conserved or non-conserved mutations are considered, millions of different sequences for the pig FSH β-subunit may exist which may, or may not, have substantial functional differences. Furthermore, claims 1 and 2 encompass the use of markers that are not even within the FSH β-subunit gene, as to the remaining claims which require the detection of an additional gene associated with pig litter size. There are no working examples of additional sequences other than those disclosed in either the specification or the prior art. The post-filing date art of Linville *et al.* exemplify the unpredictability in this area of technology. Linville *et al.* attempted to demonstrate a correlation

between a alleles in five different candidate genes and pig litter size, but were unable to do so (see abstract and results). Notably, one of these alleles was a restriction allele in allele of the FSH β-subunit gene (see Table 1). Linville *et al.* note that a previous study had demonstrated an association between an allele within the FSH β-subunit gene and pig litter size, teach that the polymorphism they studied may be in less linkage disequilibrium with the causative mutation. Thus, even within the FSH β-subunit gene, it is highly unpredictable as to whether or not a given polymorphism will be associated with pig litter size.

Although the level of skill in the art of nucleic acid analysis is high (the Ph.D. degree with laboratory experience), there is no predictability for which sequences exist which code for polymorphisms in pig FSH β-subunit genes. The quantity of experimentation that would be necessary to determine even one additional polymorphism in the pig FSH β-subunit gene is substantial. Applicants have not disclosed how one would go about detecting such polymorphisms that would be necessary to practice the claimed invention. Because there is no reason to expect that any additional polymorphism is associated with litter size and because of the very large number of possible polymorphisms, screening for additional polymorphisms that would be indicators or increased litter size would require performing and screening many, many successful breedings of pigs. There is no evidence, however, of any frequency of significant polymorphisms. Further, even in positive matches, the FSH β-subunit polymorphism may not correlate with litter size, as was found by Linville *et al.* The FSH β-subunit polymorphism may be coincident and unrelated with a different, unlinked (on the chromosome) polymorphism such as an FSH α-subunit polymorphism or a polymorphism in an undetermined gene that actually

regulates litter size. The FSH β-subunit polymorphism would not have any meaning or effect, but might appear to influence litter size due to it's close proximity to some other gene. The extreme unpredictability of polymorphisms in the art, combined with the absence of teaching in the prior and the large quantity of experimentation necessary in the art support a conclusion that undue experimentation is required to make and use the invention as broadly claimed.

11. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-6 and 10 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The invention relates to methods for screening pigs to determine those more likely to produce larger litters and/or those less likely to produce larger litters. The method steps of claims 1 and 2 require determining the presence of at least one marker linked to the FSH β-subunit allele(s) of SEQ ID NO: 1. Thus, the scope of claims 1 and 2 includes using any marker linked to instant SEQ ID NO: 1 as an indicator that a pig is more or less likely to produce a larger litter. The method steps of claims 3-6 and 10 require determining the presence of at least one FSH β-subunit allele associated with pig litter size, and the determining the presence of one

other gene associated with pig litter size. Claims 5 and 6 limit the scope of the "other gene" to the ESR gene or the OPN gene. The scope of claims 3-6 and 10 includes using any marker within the FSH β-subunit gene as an indicator that a pig is more or less likely to produce a larger litter, and further, claims 3-4 and 10 encompass the detection of any possible second gene that is associated with pig litter size.

The specification contains a single working example, demonstrating that a specific inserted retroposon allele is linked was linked to litter size. Namely, the specification demonstrates that when SEQ ID NO: 1 is present in the FSH β-subunit gene of a female pig, that pig is more likely to produce a smaller litter than when SEQ ID NO: 1 is absent in the FSH β-subunit gene of the pig (See Examples 1 and 2). The specification further speculates that it is also possible to establish linkage between specific alleles of "alternative DNA markers" and the FSH β-subunit allele disclosed in the instant specification. There is not specific teaching in the specification regarding these alternative markers, besides the speculation that they may exist. Thus, the genus of polymorphisms required to practice the claimed inventions includes any possible additional polymorphisms within the FSH β-subunit gene that is associated with pig litter size or linked to the disclosed polymorphism, or and additional "other" gene associated with pig litter size Thus, applicant has express possession of only one species in a genus which comprises at least hundreds of different possibilities.

With regard to the written description, all of these claims encompass the use of different polymorphisms from those disclosed in the specification.

It is noted that in Fiers v. Sugano (25 USPQ2d, 1601), the Fed. Cir. concluded that

> "...if inventor is unable to envision detailed chemical structure of DNA sequence coding for specific protein, as well as method of obtaining it, then conception is not achieved until reduction to practice has occurred, that is, until after gene has been isolated...conception of any chemical substance, requires definition of that substance other than by its functional utility."

In the instant application, only the specific structure of a single polymorphism has been disclosed. Also, in Vas-Cath Inc. v. Mahurkar (19 USPQ2d 1111, CAFC 1991), it was concluded that:

> "...applicant must also convey, with reasonable clarity to those skilled in art, that applicant, as of filing date sought, was in possession of invention, with invention being, for purposes of "written description" inquiry, whatever is presently claimed."

In the application at the time of filing, there is no record or description which would demonstrate conception of methods which utilize any polymorphism other than the single retroposon described in the specification.

Double Patenting

12. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington,* 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

13. Claims 1-6 and 10 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-3 of U.S. Patent No. 6291174. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '174 patent are a species of claims 1-2 and thus anticipate the claim. With regard to claims 3-6 and 10, these claims differ from the claims of the '174 patent because they recite the examination of an additional gene. However, the '174 patent discloses this as a preferred embodiment within the portion of the specification that supports claims 1-3 of the '174 patent. Therefore, it would have been prima facie obvious in view of the '174 patent to examine an additional gene because this is a preferred embodiment within the patent.

Response to Remarks

The rejections have all been modified in light of applicant's amendments. Applicant's remarks are addressed insofar as they are relevant to the pending claims.

Applicants assert in the last paragraph of page 7 that ample evidence exists in the specification as filed to demonstrate that applicants were in possession of the claimed invention. Applicants point to page 3 of the specification to support the assertion that they were in possession of additional markers of litter size that would be linked to instant SEQ ID NO: 1. However, this prophetic statement does not indicate possession of the additional markers necessary to practice the claimed invention. While it is noted that applicants are not claiming a specific DNA sequence, the knowledge of these specific DNA markers is necessary for the practice of the claimed invention. Applicant refers to the post filing date art of Slatkin *et al.* for a

general statement that markers in strong linkage disequalibirum with a disease phenotype are likely to be linked to the causative locus. However, this is not a statement that the actual markers are known by applicant or in applicant's possession. That such additional markers even exist within or near the FSH β-subunit gene is unknown at the time of filing. When Linville *et al.* were unable to detect associations between genes and litter size in particular, they stated

> "One possible reason for the lack of effect in the current study, by genes that had positive effects on litter size in other studies, is that different linkage disequilibrium existed in the populations. The polymorphisms in the genes studied may not directly affect the trait. These polymorphisms could be markers linked with the causative mutation within the gene or a closely linked gene (p. 66)."

Again, however, these linked markers are unknown and unpredictable. The postulation that additional markers exist does not demonstrate possession of the markers or provide description of them for use in the claimed methods.

With regard to the written description rejection, applicant further argues that once a polymorphism is found it would be routine to associate it with the trait. This is not persuasive for the written description rejection because it does not address the issue at hand, which is that the additional polymorphisms associated with the trait are undisclosed entirely. Further, court has made it clear that with regard to chemical compounds, the standard for written description is possession, not enablement or intent to claim. "While we have no doubt a person so motivated would be enabled by the specification to make it, this is beside the point for the question is not whether he would be so enabled but whether the specification discloses the compound to him, specifically, as something appellants actually invented. We think it does not." In Re Ruschig, 379 F.2d 990, 995, 154 U.S.P.Q. 118, 123 (CCPA 1967). Furthermore, the court stated

"Accordingly, naming a type of material generally known to exist, in the absence of knowledge as to what that material consists of, is not a description of that material." The Regents of the University of California v. Eli Lilly & Co., 43 U.S.P.Q.2d 1406 (Federal Circuit 1997). In the instant case, although applicant has provided a general function (association with litter size) this are not sufficient to convey possession of the entire possible group of methods for identifying pigs that are encompassed by the instant claims.

Conclusion

14. No claims are allowed.

15. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

16. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Juliet C Einsmann whose telephone number is (703) 306-5824. The examiner can normally be reached on Monday through Thursday, 7:00 AM to 4:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Juliet C. Einsmann can be reached on (703) 308-1152. The fax phone numbers for the organization where this application or proceeding is assigned are (703) 308-4242 and (703) 305-3014.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

Juliet C Einsmann
Examiner
Art Unit 1634

February 22, 2003

GARY BENZION, PH.D.
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

Notice of References Cited	Application/Control No. 09/855,682	Applicant(s)/Patent Under Reexamination LI ET AL.	
	Examiner Juliet C Einsmann	Art Unit 1634	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	WO-9218651	10-1992	wo	Rothschild et al.	----
	O	WO-9641892	12-1996	WO	Mileham et al.	----
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	Li et al. Biology of Reproduction. August 1997. Volume 56, Supp. p. 119, abstract #148
*	V	Rohrer et al. Mammalian Genome 5, 315-317 (1994)
	W	Linville et al. J. Anim Sci 2001. 79:60-67.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Candidate gene analysis for loci affecting litter size and ovulation rate in swine[1,2,3,4]

R. C. Linville*,[5], D. Pomp*, R. K. Johnson*,[6], and M. F. Rothschild†

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†Department of Animal Science, Iowa State University, Ames 50011-3150

ABSTRACT: A candidate gene approach was used to determine whether specific loci explain responses in ovulation rate (OR) and number of fully formed (FF), live (NBA), stillborn, and mummified pigs at birth observed in two lines selected for ovulation rate and litter size compared with a randomly selected control line. Line IOL was selected for an index of OR and embryonic survival for eight generations, followed by eight generations of two-stage selection for OR and litter size. Line C was selected at random for 16 generations. Line COL, derived from line C at Generation 8, underwent eight generations of two-stage selection. Lines IOL and C differed in mean EBV by 6.1 ova and 4.7 FF, whereas lines COL and C differed by 2.2 ova and 2.9 FF. Pigs of Generation 7 of two-stage selection lines were genotyped for the retinol binding protein 4 (*RBP4*, n = 190) and epidermal growth factor (*EGF*, n = 189) loci, whereas pigs of Generations 7 and 8 were genotyped for the estrogen receptor (*ESR*, n = 523), prolactin receptor (*PRLR*, n = 524), follicle-stimulating hormone β (*FSH*β, n = 520), and prostaglandin-endoperoxide synthase 2 (*PTGS2*, n = 523) loci. Based on chi-square analysis for homogeneity of genotypic frequencies, distributions for *PRLR*, *FSH*β, and *PTGS2* were different among lines ($P < 0.005$). Differences in gene frequencies between IOL vs C and COL vs C were 0.33 ± 0.25 and 0.16 ± 0.26 for *PRLR*, 0.35 ± 0.20 and 0.15 ± 0.24 for *FSH*β, and 0.16 ± 0.16 and 0.08 ± 0.18 for *PTGS2*. Although these differences are consistent with a model of selection acting on these loci, estimates of additive and dominance effects at these loci did not differ from zero ($P > 0.05$), and several of them had signs inconsistent with the changes in allele frequencies. We were not able to find significant associations between the polymorphic markers and phenotypes studied; however, we cannot rule out that other genetic variation within these candidate genes has an effect on the traits studied.

Key Words: Candidate Genes, Litter Size, Ovulation Rate, Pigs, Reproduction

 J. Anim. Sci. 2001. 79:60–67

Introduction

Reproductive rate, especially litter size, is one of the most economically important traits in pig production. Increasing the number of pigs weaned per sow will increase economic returns for pig producers with minimal additional inputs (Rothschild, 1996).

Currently, litter size varies from approximately 2 to 20 pigs per litter, with means from 9 to 11, depending on the breed. Phenotypic standard deviations are between 2.5 and 3 pigs, and heritability is 10 to 15% (Johnson et al., 1999). Therefore, sufficient genetic variability exists to increase litter size. However, litter size is sex-limited and selection response could be enhanced by direct selection in both sexes for genes affecting its expression.

Advances in molecular techniques can now be used to increase rate of response to selection. It has been proposed that candidate gene analyses be used to identify individual genes responsible for traits of economic importance (Rothschild and Soller, 1997). For example, Short et al. (1997) found the additive effect associated with the B allele of the estrogen receptor (***ESR***) gene was 0.42 pigs per litter in first-parity gilts. The objective of this study was to determine whether certain candi-

[1]Published as Paper No. 12981 journal ser., Nebraska Agric. Res. Div.

[2]We acknowledge the contributions of Lori Messer for supervising laboratory procedures involved in DNA analyses and Joe Cassady for assistance in collection of phenotypic data.

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date genes explain responses in ovulation rate and litter size in two lines that were selected either 16 or 8 total generations for ovulation rate, embryonic survival, and litter size.

Materials and Methods

Population. The population of pigs sampled in this experiment was developed at the University of Nebraska Swine Research Station. Three lines that originated from the Index (**I**) and Control (**C**) lines described in Johnson et al. (1999) were used. The Index and Control lines had a common base of a Landrace/Large White composite population. Pigs were randomly assigned, within litter, to either line I or line C at Generation 0 and then selected for increased values of an index of ovulation rate and embryonic survival (Line I) or randomly (Line C). At Generation 8 of index selection, the lines reported on here and designated as **IOL** and **COL** were formed from line I and line C, respectively. Eight generations of two-stage selection in lines IOL and COL were practiced. Stage-one selection included all gilts from 50% of litters with the greatest number of fully formed pigs at birth. Stage-two selection included the 50% of these gilts with the greatest ovulation rate, measured by counting the number of corpora lutea via laporatomy at second estrus. Line C continued to be randomly selected.

At Generation 0 of two-stage selection, line I, and thus line IOL, differed from line C by 4.22 ova and 1.94 pigs (Ruiz-Flores et al., 1999). After eight generations of two-stage selection, lines IOL and C differed in mean estimated breeding value by 6.1 ova and 4.7 fully formed pigs, whereas lines COL and C differed by 2.24 ova and 2.9 fully formed pigs. The inbreeding coefficients were 0.191, 0.141, and 0.137 for lines IOL, COL, and C, respectively, at Generation 8.

Measurement of Traits. Animal management and procedures to record traits are described in Johnson et al. (1999) and in Ruiz-Flores et al. (1999). All data are for first-parity females. Gilts were observed for estrus and date of final estrus was recorded. Laparotomy was performed between 8 and 14 d after gilts expressed their second estrus. Selected gilts were mated at their third or fourth estrus and numbers of fully formed, live, stillborn, and mummified pigs at birth were recorded. Data for this experiment are from pigs of Generations 7 and 8 of two-stage selection. Ovulation rate in 382 gilts and litter size data in 244 gilts were recorded.

DNA Preparation. Ear tissue of pigs in Generations 7 and 8 was collected. In Generation 7, only the selected gilts and boars that were mated were sampled (n = 192). In Generation 8, tissue was collected from all gilts in which laporatomy was performed and in all breeding boars (n = 332). Genomic DNA was purified by standard procedures using proteinase K digestion followed by phenol/chloroform extraction and precipitation with isopropanol.

Candidate Genes. Pigs were genotyped for six candidate genes (Table 1) chosen based on known physiological mechanisms or because the gene was found to be associated with litter size in other studies. Information for PCR-based genotyping for all gene markers is in Tables 1 and 2.

Primers and reaction conditions for estrogen receptor (***ESR***) were those described in Short et al. (1997). The pig specific primers and reaction conditions for epidermal growth factor (***EGF***) are described in Mendez et al. (1999). Amplification of *EGF* with the pig specific primers sometimes yielded ambiguous genotypes because the A allele in the heterozygote amplified inefficiently. A second primer pair was used to amplify only the A allele (*EGF* L1 F, Table 1) to differentiate between the AB and BB genotypes. The same reaction conditions were used for both primer pairs.

Primers for the beta subunit of follicle-stimulating hormone β (***FSHβ***) were developed from pig sequence data (Genbank accession no. D00621). Primers for prolactin receptor (***PRLR***) were developed by M. F. Rothschild (unpublished data). The AB and BB genotypes for *PRLR* were distinguishable by the intensity of the 127-bp band (Table 1), which was much darker in the AB genotype. A monomorphic band of size 35 bp comigrated with the 35-bp digestion product in the B allele.

Reaction conditions and primer sequences for prostaglandin-endoperoxide synthase 2 (***PTGS2***) are described in Gladney et al. (1999). Primers for retinol-binding protein 4 (***RBP4***) were developed by Rothschild et al. (2000) and reaction conditions are in Messer et al. (1996b).

Statistical Analysis. Allele frequencies were determined by the total count of an allele in a line divided by twice the number of observations in that line. A chi-square analysis for homogeneity of genotypic frequencies across lines was conducted in a 3×3 contingency table (Weir, 1996). The null hypothesis was that genotypic frequencies across lines were homogeneous. If a significant chi-square was obtained from Generation 7 data, then pigs of Generation 8 were also genotyped for that locus. The genotypes determined for Generation 7 animals were for the boars and gilts selected as parents of Generation 8. Thus, if genotypic frequencies among lines did not differ for parents, then differences among their progeny were not expected. Generation 8 progeny were genotyped to increase sample size if genotypic frequencies differed among Generation 7 parents.

Changes in allele frequencies between lines IOL and C and between COL and C were determined by subtraction. A standard error for the difference in gene frequency was determined by a method described in Ollivier et al. (1997) that accounted for genetic drift. The formula was: $V(\hat{p}_{gs} - \hat{p}_{gc}) = \hat{q}_{gc}\hat{p}_{gc}(1/[2n_{gc}] + F_{gc}) + \hat{q}_{gs}\hat{p}_{gs}(1/[2n_{gs}] + F_{gs})$, where $\hat{p}$ and $\hat{q}$ are estimates of gene frequencies, the subscript g represents the seventh or eighth generation, the subscripts c and s code for either the control line or a selection line, respectively, F is the inbreeding coefficient in Generation g, and n is the

Table 1. Primer sequences, restriction enzymes, allele sizes, and chromosomal locations of candidate genes

Candidate gene	Primer sequence	Enzyme	Allele size, bp			Chromosome
			A allele	B allele	Uncut	
Estrogen receptor	*ESRF* 5′ CCT GTT TTT ACA GTG ACT TTT ACA GAG 3′ *ESRR* 5′ CAC TTC GAG GGT CAG TCC AAT TAG 3′	*Pvu*II	55	65	120	1
Epidermal growth factor	Pig *EGF* F 5′ GAA ACA ATT CCC GTG TTC TCT 3′ Pig *EGF* R 5′ TCA CTT CCA CAC CTG TAA CAT CT 3′	none	1,527	625	1,527 or 625	8
	EGF L1 F 5′ TGA TCC CGT AGA AAG GTA AA 3′ *EGF* L1 R 5′ GGC ATC TAT CTG GAC AAA G 3′	none	600		600	
Retinol binding protein 4	*RBP4* F 5′ GAG CAA GAT GGA ATG GGT T 3′ *RBP4* R 5′ CTC GGT GTC TGT AAA GGT G 3′	*Msp*I	190 154 136	109 136 125	480	14
Prostaglandin-endoperoxide synthase 2	*COX2*-3F 5′ GTG CAC TAC ATA CTT ACC CAC TTC 3′ *COX2*-4R 5′ AGG CTT CCC AGC TTT T(A/G)TA 3′	*Mse*I	360[a]	240[a] 120	1,550	9
Follicle stimulating hormone β	*FSHR2**N 5′ GTA TAC CAG GTC CTA AG 3′ *FSH*-F2-RL 5′ GTC TCG TAC ACC AGC TCC TT 3′	α*Taq*I	1,650	975 675	1,650	2
Prolactin receptor	*PRLR*4 5′ CGG CCG CAG AAT CCT GCT GC 3′ *PRLR*5 5′ ACC CCA CCT TGT AAC CCA TCA TCC 3′	*Alu*I	127[a]	92[a] 35	170	16

[a]Monomorphic bands were also present.

number of animals with genotypic data per line. The line designated by s was either IOL or COL depending on the standard error being calculated (IOL-C or COL-C).

Data were analyzed using the MTDFREML program (Boldman et al., 1993) with an animal model including pedigree relationships back to the base generation of the population, year, and line as fixed effects. In one model, a covariate of the number of favorable alleles in the genotype for each gene (0, 1, or 2) was added to estimate the effects of allele substitution, and in a separate model, the effects of each genotype were estimated by fitting a fixed class variable with values of 0, 1, or 2 for the number of favorable alleles in the genotype. Only phenotypic data for Generations 7 and 8 were analyzed because genotypic data were available only for these generations. Therefore, line was included in the model to account for previous selection effects.

The genotypes of all six markers were fitted simultaneously. The favorable allele was defined as the one with increased frequency in the selection lines compared to the control line. Additive (a) and dominance (d) effects, as defined by Falconer and Mackay (1996), were estimated with orthogonal contrasts of solutions for genotypic effects. The mixed-model analysis was expected to produce unbiased estimates of a and d effects of the loci studied, averaged within line, independent of other polygenic effects on the traits (Kennedy et al., 1992).

The a and d effects of each allele also were estimated with PROC GLM of SAS (SAS Inst., Inc., Cary, NC) to determine whether solutions differed without correction for the effects of the other candidate genes and without correction for breeding values due to genes not included in the model. These analyses were performed fitting effects of each gene independently. The model included fixed effects of year and line and the appropriate genotypic classes as fixed effects.

Results

Allele frequencies are presented in Table 3. As expected for Type I markers, only two alleles for each marker existed. Both alleles for each marker were present in all three lines, although sometimes at low frequency, except for the B allele of *ESR*, which did not exist in the sample of animals of the COL and C lines. The A allele was designated as favorable for *PTGS2*

Table 2. PCR reaction conditions for the candidate genes[a]

Candidate gene	dNTP, μM each	Primer, nM each	$MgCl_2$ mM	*Taq* units	Reaction volume, μL	Genomic DNA, ng
Estrogen receptor	200	200	1.5	1.15	30	50
Epidermal growth factor	100	300	2	1.15	30	50
Retinol binding protein 4	100	320	1.5	1	20	50
Prostaglandin-endoperoxide synthase 2	200	250	1.5	0.5	10	50
Follicle-stimulating hormone β	100	200	1.5	1	20	50
Prolactin receptor	100	200	1.5	1	20	50

[a]All reactions used 1× concentration of the supplied PCR buffer and 1× RediLoad (Research Genetics, Huntsville, AL).

Table 3. Allele frequencies overall and within lines IOL, COL, and C[a]

Candidate gene	Allele	Overall	IOL	COL	C
Estrogen receptor	A	0.98	0.94	1	1
(*ESR*, n = 520)	B	0.02	0.06	0	0
Epidermal growth factor	A	0.08	0.06	0.1	0.1
(*EGF*, n = 190)	B	0.92	0.94	0.9	0.9
Retinol binding protein 4	A	0.45	0.46	0.47	0.42
(*RBP4*, n = 190)	B	0.54	0.54	0.53	0.58
Prostaglandin-endoperoxide synthase 2	A	0.91	0.98	0.9	0.82
(*PTGS2*, n = 523)	B	0.09	0.02	0.1	0.18
Follicle-stimulating hormone	A	0.2	0.03	0.23	0.38
(*FSHβ*, n = 520)	B	0.8	0.97	0.77	0.62
Prolactin receptor	A	0.33	0.19	0.36	0.42
(*PRLR*, n = 524)	B	0.67	0.81	0.64	0.58

[a]Lines IOL, COL and C are from the Nebraska selection lines. IOL was selected eight generations for index of ovulation rate and embryo survival, followed by eight generations of two-stage selection for ovulation rate and litter size. COL was selected at random for eight generations, followed by eight generations of two-stage selection. Line C was randomly selected for 16 generations.

and *RBP4*, whereas B was designated as favorable for *ESR*, *FSHβ*, *EGF*, and *PRLR*. There was an approximate linear increase in frequency of the favorable allele for *FSHβ*, *PTGS2*, and *PRLR*, genes for which the frequency of the favorable allele in line COL was intermediate to frequencies in lines C and IOL (Table 3).

Chi-square statistics for homogeneity of genotypic frequencies (Table 4) for *FSHβ*, *PTGS2*, *PRLR*, and *ESR* were significant. It is likely that the chi-square statistic for *ESR* is significant because there were several zeros in the observational cells of the contingency table. Small values (less than 5) in individual cells of the contingency table inflate the chi-square value (Weir, 1996). Thus, the chi-square test for the *ESR* locus is not accurate and we cannot conclude that genotypic frequencies differ across lines.

Changes in allele frequencies are presented in Table 5. After accounting for potential genetic drift, none of the differences in allele frequencies between selection lines and line C was significantly different from zero.

None of the estimates of the effects of allele substitution differed significantly from zero. These results are not presented because they can be calculated with formulas found in Falconer and Mackay (1996) using estimates of gene frequencies (Table 3) and a and d effects (Table 6).

Estimates of the additive and dominance effects of alleles calculated with an animal model are in Table 6. Only the estimate of dominance effect of *PTGS2* on number of mummies differed from zero. Estimates of a and d effects calculated without an animal model (not shown) were very similar to those in Table 6.

Discussion

Choice of Candidate Genes. The estrogen receptor locus was chosen because results from Rothschild et al. (1996) and Short et al. (1997), using data from nearly 10,000 litters from several lines, reported that the *ESR* B allele was significantly associated with increased number of pigs per litter at birth. Estrogen is involved in maternal recognition of pregnancy (Geisert et al., 1990). It is produced by the growing conceptus and is

Table 4. Observed numbers of genotypes in lines IOL, COL, and C and chi-square tests of homogeneity of genotypic frequencies[a]

Genotype	IOL	COL	C	χ^2
		ESR		
AA	166	198	136	
AB	22	0	0	
BB	1	0	0	42.5***
		EGF		
AA	0	0	0	
AB	8	13	11	
BB	58	55	45	2.7
		RBP4		
AA	8	15	12	
AB	46	35	21	
BB	13	19	21	12.3
		PTGS2		
AA	181	162	94	
AB	8	33	36	
BB	0	2	7	47.2***
		FSHβ		
AA	0	8	16	
AB	12	73	70	
BB	176	115	50	124.7***
		PRLR		
AA	4	23	35	
AB	64	97	73	
BB	121	78	29	79.6***

[a]AA, AB, and BB are the genotypes for the markers estrogen receptor (*ESR*), epidermal growth factor (*EGF*), retinol binding protein (*RBP4*), prostaglandin endoperoxide-synthase 2 (*PTGS2*), follicle-stimulating hormone (*FSHβ*), and prolactin receptor (*PRLR*). The expected values were calculated by dividing the total for a genotype by the grand total, then multiplying by the total for each line. IOL was selected eight generations for index followed by eight generations of two stage selection, COL was selected randomly for eight generations followed by eight generations of two-stage selection, and C was randomly selected for 16 generations.

***$P < 0.001$.

Table 5. Differences in allele frequencies of lines IOL and COL from C[a]

Candidate gene	IOL-C	SE w/drift	SE w/o drift	COL-C	SE w/drift	SE w/o drift
Estrogen receptor (*ESR*, n = 520)	0.06	0.116	0.039	0	0.116	0.039
Epidermal growth factor (*EGF*, n = 190)	0.04	0.156	0.035	0	0.156	0.038
Retinol binding protein 4 (*RBP4*, n = 190)	0.04	0.29	0.063	0.05	0.27	0.063
Prostaglandin-endoperoxide synthase 2 (*PTGS2*, n = 523)	0.16	0.156	0.007	0.08	0.18	0.0005
Follicle-stimulating hormone (*FSHβ*, n = 520)	0.35	0.2	0.03	0.1a	0.24	0.035
Prolactin receptor (*PRLR*, n = 524)	0.33	0.25	0.038	0.16	0.26	0.038

[a]IOL was selected eight generations for index of ovulation rate and embryonic survival, followed by eight generations of two-stage selection for ovulation rate and litter size. COL underwent two-stage selection for eight generations, subsequent to eight generations of random selection. Line C was randomly selected for 16 generations.

recognized by receptors in the uterus of the sow. Also, estrogen acts to induce hypertrophy and hyperplasia of the myometrial cells (Hafez, 1993).

Epidermal growth factor was chosen based on its role in the physiology of reproduction. Epidermal growth factor is produced by the conceptus and in the uterus of the sow. In the fetus, *EGF* stimulates growth and proliferation of skin epithelia; in the neonate, *EGF* stimulates pulmonary epithelia to grow and mature (Hadley, 1996).

Follicle-stimulating hormone is a heterodimer composed of alpha and beta subunits that are coded by two distinct genes. The beta subunit offers specificity. Follicle-stimulating hormone was chosen as a candidate gene because it functions in maturation of small and medium follicles into large follicles that ovulate (Wang and Greenwald, 1993a,b; Mannaertz et al., 1994). Also, in a candidate gene analysis, Li et al. (1998) found major effects on litter size associated with a marker within *FSHβ*.

The prolactin receptor locus was chosen as a candidate gene because Vincent et al. (1998) reported that it was associated with litter size in three of the five lines of pigs they studied. Prolactin affects production of progesterone and relaxin from the corpora lutea, as determined by comparison of hypophysectomized gilts with and without prolactin treatment vs sham-operated gilts (Yangfan et al., 1989). It was inferred in that study that prolactin is leuteotropic during the latter part of pregnancy.

Prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase 2 (***COX2***), was chosen because it is the rate-limiting enzyme in the formation of prostaglandins (Lim et al., 1997). A null mutation that produced

Table 6. Additive (a) effects of favorable[a] allele and dominance (d) effects estimated with an animal model[b,c]

Candidate gene	Contrast		OR	SE	FF	SE	NBA	SE	Stillborn	SE	Mummies	SE
ESR	BB-AA	a	0.108	1.3	1.74	1.6	0.474	1.52	1.25	0.761	0.341	0.437
		d	2.33	1.42	2.72	1.98	1.58	1.88	1.13	0.933	0.37	0.54
RBP4	AA-BB	a	−0.284	0.38	0.179	0.457	0.526	0.436	−0.346	0.22	−0.026	0.124
		d	0.315	0.49	0.441	0.627	0.313	0.595	0.0936	0.298	−0.0479	0.17
PTGS2	AA-BB	a	0.036	0.64	0.589	0.833	0.403	0.795	0.184	0.399	0.273	0.226
		d	0.448	0.71	0.354	0.953	0.076	0.909	0.278	0.454	0.741*	0.259
FSHβ	BB-AA	a	−0.04	0.34	0.163	0.466	0.12	0.446	0.045	0.223	0.246	0.127
		d	−0.039	0.41	0.979	0.577	0.759	0.549	0.222	0.273	0.0481	0.157
PRLR	BB-AA	a	−0.287	0.27	−0.039	0.380	−0.007	0.366	−0.028	0.184	0.091	0.103
		d	−0.445	0.32	−0.229	0.462	−0.466	0.440	0.164	0.219	0.063	0.126

[a]Favorable allele was defined as the one whose frequence increased in lines IOL and COL relative to line C. The additive contrast (a) is the favorable genotype minus the less favorable genotype, and d = AB − 1/2(AA + BB).

[b]Epidermal growth factor (*EGF*) could not be estimated with contrasts because only two genotypes occurred.

[c]Traits measured include ovulation rate (OR), number of fully formed (FF), live (NBA), stillborn, and mummified pigs. Candidate genes were *estrogen receptor* (*ESR*), *retinol binding protein* (*RBP4*), *prostaglandin endoperoxide-synthase 2* (*PTGS2*), *follicle-stimulating hormone* (*FSHβ*), and *prolactin receptor* (*PRLR*).

multiple reproductive failures in mice was described (Lim et al., 1997). Oocyte maturation was not complete, and the first polar body was usually not extruded. Also, when wild-type blastocysts were implanted into the uteri of mutant mice, they failed to implant.

Retinol-binding protein 4 was studied as a possible candidate gene affecting litter size because it is involved in embryonic development. Yelich et al. (1997) stated that most embryonic death losses occur between d 10 and 18 of gestation, concurrent with trophoblast elongation and secretion of estrogen by the conceptus. Retinol-binding protein 4, a major protein produced by the conceptus, may have a role in trophoblast elongation (Harney et al., 1990). It also enhances gene expression of transforming growth factor β via retinoic acid receptors (Yelich et al., 1997). Rothschild et al. (2000), using data of 2,500 litters of six commercial lines, reported an additive effect associated with *RBP4* of 0.23 pigs/litter.

Allele Frequencies and Effects. The magnitude of the differences in allele frequencies between IOL and C and between COL and C were small (less than 0.06) for markers within the *ESR*, *RBP4*, and *EGF* loci. The differences in allele frequencies were greater for the *FSHβ*, *PRLR*, and *PTGS2* loci. Within these latter three loci, there was an approximately linear increase in frequency of the favorable allele between the lines that were separated by eight generations of selection (COL and C) and the lines separated by 16 generations of selection (IOL and C).

The effects of selection, different frequencies in the founder animals, and random genetic drift could have caused allele frequencies to differ among lines. The base generation was sampled to minimize founder effects. The I and C lines were created from a common base population in which littermates were assigned at random to either the I or C line. All base generation parents were represented in both lines.

Random genetic drift is a nondirectional force that acts to change allele frequencies. In the absence of selection, the amount of drift depends on allele frequencies and effective population size. The estimated inbreeding coefficients at Generation 16 were used to estimate effective population sizes (N = 38, IOL; 53, COL; and 54, C). For alleles with frequencies between 0.1 and 0.5, these values of N result in estimates from 0.029 to 0.057 for SE of change in allele frequency within lines in any generation due to drift. After drift begins, it is most probable that an allele's frequency will continue to change in the same direction (Falconer and Mackay, 1996). Thus, drift could have caused the linear increase in frequencies of favorable alleles at the *PTGS2*, *FSHβ*, and *PRLR* loci. Knowledge of base generation allele frequencies is needed to calculate the probability that random drift alone caused the order of favorable allele frequencies to be C, COL, to IOL at each of these three loci.

The fact that frequencies of favorable alleles at three loci are approximately linearly ordered according to number of generations of selection also fits a selection model. Equations from Falconer and Mackay (1996) for an additive model were used to determine values of the selection coefficient (s) against the homozygous genotypes for the unfavorable alleles at each of the *PTGS2*, *FSHβ*, and *PRLR* loci. Assuming allele frequencies in the control line represent the base generation, values of s that would have produced observed allele frequency changes in line IOL are 0.13, 0.25, and 0.21 for the *PTGS2*, *FSHβ*, and *PRLR* loci, respectively. However, even with selection, allele frequencies at these loci were subject to genetic drift. With selection, the amount of drift depends approximately on the product of effective population size and the selection coefficient at that locus (Hartl and Clark, 1989). For effective population size of N and selection coefficient of s against the least favorable homozygous genotype, selection has little effect on rate of genetic drift if $Ns < 1$. Thus, selection coefficients greater than 0.025 would have slowed the rate of increase in the frequency of an unfavorable allele in lines IOL and COL and would have speeded the rate of increase of a favorable allele. With drift, selection coefficients less than these calculated values could have produced the same changes in allele frequencies.

The joint effects of selection and drift as forces for change in allele frequencies in this study cannot be separated. When inbreeding coefficients were incorporated into the variances of allele frequency differences among lines, the estimates of standard errors were increased in the range of three to seven times (Table 5). Changes in allele frequencies were not significant, although differences calculated without including drift variance were significant. Thus, a selection model cannot be ruled out as the cause of the linear increase in allele frequencies at the *PTGS2*, *FSHβ*, and *PRLR* loci from lines C to COL to IOL (Table 3).

When selection lines have accumulated inbreeding of the levels in these lines, changes in frequency for any allele will not be significant, and evaluating changes in gene frequency by itself is not informative. However, allele frequency changes that are consistent with additive and dominance effects give greater evidence that selection caused the changes.

The linear regression of phenotype on genotype, fitting all marker genotypes simultaneously and using an animal model to account for the background genotypes and fixed effects of year and line, yielded no significant estimates of allele substitution effects. At several loci, including the *FSHβ* and *PRLR* loci for which there were linear increases in allele frequencies, the estimate of the effect of the favorable allele was negative. This finding does not support a selection model operating at these loci. In addition, using estimates of allele frequency changes and the a and d effects, we calculated that only 2 to 5% of the total responses in ovulation rate and litter size could be explained by allele frequency changes at all loci studied.

The additive and dominance effects were estimated both with and without an animal model. Estimates were nearly identical from both analyses. Only one ef-

fect was found to be significantly different from zero, the dominance effect of *PTGS2* on the number of mummies. Standard errors were approximately 10% greater for effects estimated with an animal model, consistent with the results of the simulation study of Kennedy et al. (1992).

Finding no significant effects for any of the genes on any of the traits was an unexpected result, based on results of other studies. Messer et al. (1996a) reported effects associated with *RBP4* to be 0.52 ± 0.45 for litter size in hyperprolific Large White sows in France and 0.32 ± 0.30 for control sows; Rothschild et al. (2000) reported smaller effects in over 2,000 litters from across several populations. Retinol binding proteins are synthesized just before elongation of the trophoblast (Yelich et al., 1997). This timing of transcription and the role of *RBP4* in embryo development implicate it as a candidate gene for litter size. However, it did not seem to explain response to selection in our lines.

The inconsistency across lines of the effects of an allele is evident from the work reported by Vincent et al. (1998). They reported the *PRLR* marker was linked to a significant additive effect on litter size in three of five PIC lines. Significant dominance effects occurred in two of the three lines for number born alive, but they were opposite in sign (d = –0.33, $P < 0.1$; d = 0.63, $P < 0.01$). Significant additive effects also existed in the same two lines for total number born ($P < 0.05$; range from a = 0.16 to a = 0.51) and the dominance effect was also important in one line ($P < 0.05$, d = 0.55). In one line, the additive effect for number born alive was –0.33, whereas it was positive (0.47) in two other lines. There were more records in the data reported by Vincent et al. (1998) than in the current study. The lines with significant effects mentioned above had from 261 to 416 animals per line, and some sows had multiple records, giving a total of 685 to 1,197 records per line. The variation among lines in that study and the different results in the current study could be due to sampling. Short et al. (1997) stated that over 1,000 records were needed before a stable estimate of 0.42 pigs per litter in first-parity gilts of the additive effect of the B allele of the *ESR* gene occurred.

The *ESR* locus did not explain response to selection in the IOL and COL lines. The gene likely was segregating in the base generation because both the A and B alleles existed in Line IOL. However, the frequency of the B allele, defined as the favorable allele (Short et al., 1997), was 0.06 after 16 generations of selection. The B allele was lost due to random drift in line C, presumably before the eighth generation when Line COL was formed because it did not exist in either Lines C or COL.

The candidate gene analysis of Li et al. (1998), in which a significant difference of 2.53 pigs per litter for alternative homozygotes for two alleles of *FSHβ* was found, used a polymorphism different from the one we studied. The polymorphism we studied may be in less linkage disequilibrium with the causative mutation.

Cassady et al. (2000) found greater concentrations of circulating FSH in the lines selected for increased ovulation rate than in their randomly selected controls. However, the *FSHβ* gene does not seem to explain response to selection in Lines IOL and COL. It is important to stress that the lack of association between a polymorphism within a gene and a phenotype does not mean that the gene product is not important in regulating the trait. Effects of transcriptional and(or) translational events at other loci may combine to regulate FSH levels in these selection lines of pigs. Indeed, a recent QTL scan for FSH levels in pigs identified several chromosomal regions, independent of the *FSHβ* locus, influencing this trait (G. Rohrer, personal communication).

One possible reason for the lack of effect in the current study, by genes that had positive effects on litter size in other studies, is that different linkage disequilibrium existed in the populations. The polymorphism in the genes studied may not directly affect the trait. These polymorphisms could be markers linked with the causative mutation within the gene or a closely linked gene. Different linkage relationships may be the reason estimates of the effects of genes differ across populations.

Background effects of other genes and interactions of these genes with the markers can also cause estimates of gene effects to differ across populations. Little is known about the magnitude of epistatic variation in the traits studied here. But if epistasis exists, the effect of a particular allele depends on its frequency and the frequency of alleles at other loci. Because of epistasis, polymorphisms for one gene could have a small effect in one population, yet explain a significant portion of the variance in another population.

Cassady (1999) found results consistent with those of this study. He performed a complete genome scan for QTL affecting reproduction traits in an F_2 population of the Nebraska Index and Control lines that were the foundation lines for those used here. He found evidence ($P < 0.05$) of a QTL on chromosome 11 affecting number of pigs per litter, one QTL on each of chromosomes 5 and 13 affecting number of stillborn pigs per litter, and one QTL on chromosome 9 affecting ovulation rate. The *PTGS2* locus, located on chromosome 9, is the only one of the candidate genes that we studied that is located on one of these chromosomes. However, the QTL for ovulation rate found by Cassady (1999) was at approximately the 1 cM position, whereas *PTGS2* has been mapped between markers S0295 and S0114, which are at approximately positions 100 and 123 cM, respectively, on the USMARC linkage map (Gladney et al., 1999). Thus, results of the QTL genome scan support the conclusion that the candidate genes studied here do not have large effects on ovulation rate or litter size in this population.

Implications

Six candidate genes for effects on ovulation rate and litter size were studied in lines separated by 8 and 16

generations of selection. The selection lines exceeded the control line by 20 to 50% in ovulation rate and litter size at birth. None of the markers studied explained a significant portion of this response. Line differences in frequencies of alleles of these markers were observed. Random genetic drift likely caused these differences, but effects of selection cannot be ruled out. The selection response was likely due to small changes in the frequencies of several genes. Other studies have reported that the candidate genes studied affect litter size. However, direct selection for alleles of these genes may not increase litter size in all populations.

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(57) Abstract

Methods for screening pigs to determine which are more likely to produce larger litters and/or are less likely to produce larger litters are provided, based on identification of OPN alleles present in a sample of pig genomic DNA. Kits for use in such methods are also provided.

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DNA MARKERS FOR PIG LITTER SIZE

The present invention relates to methods of screening pigs to determine the presence or absence of osteopontin (OPN) alleles associated with increased litter size, to the use of such methods in predicting litter size in pigs and to kits for carrying out such methods.

Meat production and animal breeding efficiencies could be improved if it were possible to increase animal litter sizes. The same output of livestock could be derived from fewer parent animals, thus providing decreased production costs. In addition, animal breeding organisations would benefit from the potential to screen more offspring for those with improved genetics. However, litter size is very difficult to select for conventionally as it is limited to one sex and is heavily influenced by non-genetic factors (heritability, a measure of the fraction of the phenotypic variation that is due to genetic differences is approximately 0.1 for litter size in the pig).

One approach to improving litter size might be to introduce beneficial genes into production lines from breeds which have significantly higher litter sizes. However, quantitative genetics suggests that complex traits such as litter size are controlled by a large number of genes each having a small effect on the trait. If this is true, genetic progress through selection of complex traits is likely to be very slow. An alternative view is that, although many genes are involved in complex traits, a few of the genes involved (major genes) have large effects on the trait. If this alternative view is true, then genetic progress of such traits could be

rapid, provided that it is possible to identify and select for beneficial alleles of relevant major genes. Since the advent of genome mapping, it has become possible to identify genes affecting quantitative traits (quantitative trait loci, QTL) by looking for associations between the trait and molecular markers distributed evenly across the genome of animals for which maps are available. Importantly, for selection purposes, the heritability of such marker phenotypes is 1.0.

The Chinese Meishan breed of pig is known to produce about 4 extra piglets per litter than the most prolific European breeds. Genes for prolificacy (litter size) from this breed would be of great value in programmes aimed at increasing the litter size of commercial Western pig breeds. Indeed a genetic marker associated with the oestrogen receptor gene (ESR) of the Meishan has been shown to have beneficial effects on litter size and is described in WO92/18651.

The Booroola Merino breed of sheep is extremely prolific. Litter sizes of three or more are common. The significantly increased prolificacy of this breed has been shown to be due to the action of a single gene, FECB (for review see G W Montgomery, *et al*, *Endocrine Reviews*, **13**: 309-328 (1992)). Genetic mapping using human DNA markers has shown that the human version of FECB is located on chromosome 4 (G W Montgomery, *et al*, *Nature Genetics*, **4**: 410-114 (1993)) and is closely associated with the gene encoding secreted phosphoprotein-1 (SPP-1), also known as osteopontin (OPN), 2ar, bone sialoprotein-1, 44 kDa bone phosphoprotein and tumour secreted phosphoprotein. Comparative mapping (H Ellegren, *et al*, *Genomics*, **17**: 599-603 (1993) has shown that human

chromosome 4 and porcine chromosome 8 are highly similar (syntenic). The porcine SPP-1 gene is also located on chromosome 8.

More recently, it has been shown that a FECB-linked marker in cattle does not act as a marker for increased litter size in herds selected for increased ovulation rate (Blattman *et al*, *Mid-West Animal Science Meeting*, **18**: 43 (1995)).

However, we have surprisingly found that, in pigs, certain DNA markers for OPN are associated with litter size, and thus can be used to select for pigs with a greater chance of producing increased litter size and to select against pigs which have alleles indicating smaller litter sizes. As used herein "increased litter size" means a significant increase in litter size above the mean of a given population.

It is interesting to note that there is an apparent break point in the chromosome synteny around OPN between sheep, cattle and man on the one hand and mouse and pig on the other (Montgomery *et al*, *J. Reproduction and Fertility supplement*, **49**:113-121 (1995)). This suggests that the structure of the chromosome may be altered in this region, between animals having large litters (mouse and pig) and those with small litters (man, sheep and cow), such that the effect of the major gene for fecundity is modified. Possible explanations include the expression of the major gene may have been increased or decreased by being brought into a more transcriptionally active or inactive region; the major gene may have been brought directly under the control of an altered promoter element; the position of the major gene relative to OPN

may have been changed such that OPN becomes a more useable marker in assessing litter size potential in the pig than in sheep or cattle.

Thus, in a first aspect, the present invention provides a method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:

(i) obtaining a sample of genomic DNA from a pig; and

(ii) analysing the genomic DNA obtained in (i) to determine which OPN allele(s) is/are present.

Suitably, step (ii), namely the determination of OPN alleles, is carried out by looking for particular DNA markers linked either directly or indirectly to OPN.

Association between genetic markers and genes responsible for a particular trait can be disrupted by genetic recombination. Thus, the closer the physical distance between the marker and the gene in question, the less likely it is that recombination will separate them.
It is also possible to establish linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with a particular gene (e.g. the OPN gene discussed herein), which have previously been shown to be associated with a particular trait. Thus, in the present situation, taking the OPN gene, it would be possible, at least in the short term, to select for pigs likely to produce larger litters, or

alternatively against pigs likely to produce smaller litters, indirectly, by selecting for certain alleles of an OPN associated marker through the selection of specific alleles of alternative chromosome 8 markers. Examples of such markers known to be linked to OPN on porcine chromosome 8 include Sw61, Sw1085, Sw194, Sw16, SW790 and SO178, which markers are all microsatellites.

In a further embodiment of the invention a number of such markers are used. For example, pairs of markers might be utilised to bracket the major gene to reduce any possible effects of recombination. Examples of such combinations of markers include SO178 and SW61 and SO178 and SW790.

Since the effect may be related to the difference in gene orders of pigs (and mice) and sheep (and humans and cattle), this suggests that the most useful second marker will be in the non-homologous (non-syntenic) region of pig chromosome 8. An example of a suitable combination of markers known to bracket this region would be OPN and SO178. However, the skilled man will appreciate that other useful markers could routinely be identified.

A particular genetic marker associated with OPN is a microsatellite. These are simple sequence repeats of 4, 3 or, more usually, 2 nucleotides, which occur essentially at random around the genome at approximately every 50,000 bases (about 60,000 microsatellites per haploid genome). Stuttering of DNA polymerase during replication and unequal crossing-over during recombination are thought to result in the loss or gain of repeat units. This means that microsatellites are usually polymorphic and can have several repeat length alleles.

An example of a microsatellite associated with a given gene is $(CA)_n$, resulting in possible repeat unit length alleles, e.g. $(CA)_2$, $(CA)_9$, $(CA)_{10}$, $(CA)_{11}$ and $(CA)_{12}$.

Using primers capable of hybridising (for example, under stringent conditions) to regions flanking the microsatellite associated with the given gene, in combination with standard PCR techniques, PCR products of differing lengths can be generated, the length being dependent on the particular repeat unit length allele of the microsatellite.

Analysing the association of such PCR products using the microsatellite associated with the OPN gene with litter size has allowed marker length alleles associated with increased, and decreased, litter size to be determined in pigs.

Suitable primer pairs which will hybridise to flanking regions of such microsatellites include those having the following sequence:

GCTAGTTAATGACATTGTACATAA; or
CCAATCCTATTCACGAAAAAGC; and
GTGTCATGAGGTTTTTTCCACTGC; or
CAACCCACTTGCTCCCAC.

In particular, repeat unit length alleles for the above-noted microsatellite marker, designated 132 and 136, have been found to be associated with increased litter size in pigs. In addition, the repeat unit length allele, designated 112, has been found to be associated with reduced litter size in pigs.

In fact, the allele associated with increased litter size predominantly derived from a European parent stock. This is contrary to expectations since, as discussed above, the Meishan has four extra piglets per litter than either Landrace or Duroc, and it might have been expected that beneficial markers would have been associated with genes inherited from the Meishan parent stock.

In a second aspect the present invention provides a method of screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:

(i) obtaining a sample of genomic DNA from a pig;

(ii) hybridising the genomic DNA from (i) with one or more suitable primers;

(iii) carrying out one or more PCR cycles using the hybridised nucleic acid from (ii); and

(iv) analysing the length of the PCR product obtained in (iii).

Suitably, the methods of the present invention are carried out using reagents and instructions presented in the form of a kit.

Thus, in a third aspect, the present invention provides a kit for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which comprises one or more

reagents or materials capable of identifying OPN alleles in a sample of pig genomic DNA.

A preferred kit of the invention will comprise reagents or materials capable of identifying alleles associated with DNA markers linked to the OPN gene, eg the microsatellite marker. Such a kit would most preferably comprise one or more DNA primers optionally together with standard PCR reagents.

Finally, the skilled person will realise that the methods and kits described herein can be used in conjunction with other already described methods and kits to screen pigs to determine those more likely to produce larger litters (or those less likely to). An example of such other methods and kits are those described in WO92/18651. It would, of course, be possible to produce combined kits which could be used to screen pig DNA using both methods.

In WO-A-9218651 and USSN 08/312312 there are disclosed methods for determining which pigs are more likely to produce larger litter sizes based on a linkage with the ESR gene. The skilled man will appreciate, therefore, that the screening methods of the present invention can be combined with the earlier disclosed ESR screening methods to provide a yet more powerful tool for such determinations. Thus, in a further aspect, the present invention provides a method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:

(i) obtaining a sample of genomic DNA from a pig;

(ii) analysing the genomic DNA obtained in step (i) to determine which OPN allele(s) is/are present; and

(iii) analysing the genomic DNA obtained in step (i) to dtermine which alele(s) of at least one other gene linked to litter size in pigs is/are present.

In one preferred embodiment of this aspect of the invention the at least one other gene is the ESR gene, as described in WO-A-9218631 and USSN 08/312312.

In a final aspect the present invention provides a kit for screening pigs to dtermine those more likely to produce larger litters, and/or those less likely to produce larger litters, which comprises one or more reagents or materials capable of identifying OPN alleles in a sample of pig genomic DNA, together with one or more reagents or materials capable of identifying alleles of at least one other gene linked to litter size in pigs in a sample of pig genomic DNA.

Preferred features of each aspect of the invention are applicable to each other aspect *mutatis mutandis*.

The invention will now be described with reference to the following examples, which should in no way be construed as limiting the invention

EXAMPLE 1

DNA Preparation

DNA can be prepared from any source of tissue containing cell nuclei, for example white blood cells, hair follicles, ear notches and muscle. The procedure outlined here relates to blood cell preparations; other tissues can be processed similarly by directly suspending material in K buffer and then proceeding from the same stage of the blood procedure. The method outlined here produces a cell lysate containing crude DNA which is suitable for PCR amplification. However, any method for preparing purified or crude DNA should be equally effective.

Blood should be collected in 50 mM EDTA pH 8.0 to prevent coagulation. 50 µl of blood was dispensed into a small microcentrifuge tube (0.5 ml Eppendorf or equivalent). 450 µl of TE buffer was added to lyse the red blood cells (haem groups inhibit PCR) and the mix vortexed for 2 seconds. The intact white and residual red blood cells were then centrifuged for 12 seconds at 13,000 g in a microcentrifuge. The supernatant was removed by gentle aspiration using a low pressure vacuum pump system. A further 450 µl of TE buffer was then added to lyse the remaining red blood cells and the white blood cells collected by centrifugation as before. If any redness remained in the pellet, this process was repeated until the pellet was white. After removal of the last drop of supernatant from the pelleted white blood cells, 100 µl of K buffer containing proteinase K was added and the mixture incubated at 55°C for 2 hours. The mixture was then heated to 95-100°C for 8 minutes and the DNA lysates

stored at -20°C until needed.

Reagents

TE buffer:	10 mM TRIS-HCl pH 8.0
	1 mM EDTA
K buffer:	50 mM KCl
	10 mM TRIS-HCl pH 8.3
	2.5 mM $MgCl_2$
	0.5% Tween 20

Prior to use for lysates 10 μl of 20 mg/ml proteinase K (Boehringer Mannheim) per 1.0 ml of K buffer was added.

PCR

Reactions were set up as follows in thin walled 0.25 ml tubes (Perkin Elmer):

1.5 μl 10x buffer;
1.5 μl 15 mM MgCl;
1.5 μl 2 mM dNTPs (Pharmacia);
0.5 μl of each primer at 5 mM (Genosys);
9 μl sterile deionised water;
0.1 μl (0.5 units) AmpliTaq DNA polymerase (Perkin Elmer);
1 μl DNA lysate.

Reaction tubes were then placed on a Perkin Elmer 9600 thermal cycler and PCR carried out according to the regime indicated below:

94°C for 4 minutes;
30 cycles of 94°C for 30 seconds, 58°C for 1 minute; and 72°C for 1 minute;

72°C for 4 minutes;
4°C until required.

Reagents

10x PCR buffer		100 mM Tris-HCl pH 8.3 (25°C), 500 mM KCl
Forward primer		GCTAGTTAATGACATTGTACATAA
	or	CCAATCCATTCACGAAAAAGC
Reverse primer		GTGTCATGAGGTTTGTGCCACTGC
	or	CAACCCACTTGCTCCCAC

If one of the primers is labelled with a fluorescent marker, the resulting products can be analysed on an automated DNA sequencer such as the Applied Biosystems 373 DNA Sequencer using Genescan and Genotyper software.

EXAMPLE 2

Polyacrylamide Gel Electrophoresis

5 μl of the PCR products were mixed with 2 μl of loading buffer and separated on a non-denaturing polyacrylamide slab gel in 1 x TBE buffer at 100V for 4 hours. The gel was then stained in a 50 ng/ml solution of ethidium bromide for 30 minutes and the PCR products visualised and photographed on a UV light transilluminator. PCR product sizes in base pairs were then estimated from relative mobilities as compared with known molecular weight markers run on the same gel. The size estimate of PCR products reflects the length of the microsatellite allele.

PCR products were also analysed on an Applied Biosystems DNA Sequencer following the use of a fluorescently

labelled primer in the PCR.

RESULTS

OPN Allele Frequencies

Results for OPN allele frequencies in different pig populations are presented in Table 1.

TABLE 1 **OPN Allele Frequencies in Different Pig Populations.**

Population	OPN Allele	Number	Percentage
Landrace	112	3	21
	132	6	43
	136	2	14
	142	3	21
Meishan	132	2	17
	140	8	67
	142	1	8
	154	1	8
L93	112	10	3
	122	2	1
	124	30	8
	132	39	10
	134	1	0
	136	36	9
	140	171	43
	142	60	15
	153	2	1
	154	43	11
L94	124	45	28
	132	15	9
	136	9	6
	140	84	52
	154	9	6

L93 Animals from a population founded by a Landrace x Meishan cross.

L94 Animals from a population founded by Duroc x Meishan cross.

Statistical Analysis

Female animals derived from L93 and L94 were scored for litter size (both total number born (TNB) and number born alive (NBA)), over several parities if possible, and these data were compared with OPN microsatellite genotypes for the same animal set. Statistical associations between litter size and OPN genotypes were investigated using the method of least squares to fit a general linear model. Least Squares Means (LSMs) for litter size were estimated for each OPN genotype. LSMs are the means adjusted for other effects in the model which could affect litter size.

The effect of individual OPN alleles was further dissected using an allele substitution model in which animals were classified into groups depending on whether they carried 0, 1 or 2 copies of a particular allele. LSMs for litter size were estimated for each group. The results for L93 are shown in Table 2.

TABLE 2 Allele Substitution Data for L93.

		Number of Copies of Alleles							
		0		1		2			
Allele	Trait	LSM	n	LSM	n	LSM	n	Model	OPN
112	TNB NBA	13.0 11.8	306	11.8 10.9	21		0	+ NS	+ NS
124	TNB NBA	12.9 11.8	281	13.0 11.6	46		0	+ NS	NS NS
132	TNB NBA	12.8 11.5	260	13.3 12.3	57	14.3 13.9	10	+ NS	NS *
136	TNB NBA	12.9 11.8	271	12.9 11.6	53	17.5 11.1	3	* NS	+ NS
140	TNB NBA	12.7 11.5	81	13.1 11.9	175	12.8 11.6	71	+ NS	NS NS
142	TNB NBA	13.0 11.8	253	12.8 11.7	68	11.3 9.8	6	+ NS	NS NS
153	TNB NBA	12.9 11.8	320	13.0 10.9	7		0	+ NS	NS NS
154	TNB NBA	13.0 11.8	284	12.5 11.4	39	11.5 10.7	4	+ NS	NS NS

Significance level: *, P<0.05; +, P<0.10; NS, P>0.10.

Model includes season, AI or natural service, parity, generation and OPN genotype.

TNB total number born
NBA number born alive
LSM least squares means
n number of records, i.e. litters

It can be seen from the data that allele 112 appears to be related to a negative effect on litter size, whereas positive trends are seen for alleles 132 (NBA) and 136 (TNB). The data presented in Table 1 suggests that while alleles 112 and 136 were probably derived from the Landrace, allele 132 could have been derived from either the Landrace or Meishan ancestry. However, as the 132 allele is more than twice as common in the Landrace as the Meishan, it is likely that a significant proportion of the 132 alleles in L93 derive from the Landrace.

In order to investigate the potential of these alleles to act as predictors of litter size, additional data from L94 were included in the analysis. Allele 112 was not found in this line (presumably this allele is not found in Duroc). The combined data for alleles 132 and 136 are shown in Table 3.

TABLE 3

		Number of Copies of Alleles							
		0		1		2		Significance of	
Allele	Trait	LSM	n	LSM	n	LSM	n	Model	OPN
132	TNB NBA	12.2 10.9	375	12.9 11.9	82	14.2 13.5	12	*** **	+ **
136	TNB NBA	12.3 11.1	393	12.3 11.0	73	16.9 10.5	3	*** NS	+ NS

Significance levels: ***, P<0.001; **, P<0.01; +, P<0.05; NS, not significant.

These data show that only allele 132 had a significant positive effect for litter size for both TNB and NBA. Although allele 136 was close to significance for TNB, it is probable that the effect here is due to a small amount of 136/136 animals (3) with very high observations.

The association between OPN allele 132 and high litter size has now been demonstrated in two different lines of pig (L93 and L94). This indicates that a QTL affecting litter size is closely associated with the porcine OPN gene. However, it is possible that in other families, lines or breeds of pig that a different OPN allele will be associated with increased litter size.

The results of a re-analysis of the data for L93 and L94 and for an additional line L07 (a large white line) is shown below in table 4 using an alternative model. This involved fitting each OPN allele as a variable and coding each animal with a 0, 1 or 2 for each allele (ie 0, 1 or 2 copies of each allele).

Fixed effects were herd-season-service type and parity. Sire was included as a random effect. ESR and OPN were fitted as covariables. All data per line were included, not just full- or half-sib families. OPN alleles with less than 10 litters of a second genotype were excluded from the analyses.

Traits analysed were total number born (TNB) and number born alive (NBA).

Three models were run for each line including the fixed, random and ESR effects as given above.

1. Model excluding OPN

2. Model including all OPN alleles
3. Model including OPN alleles individually

-2logliklihood was obtained for each model. Significance of the model was calculated by subtracting the log liklihood from models 2 or 3 from model 1 and comparing the result against a Chi-squared distribution. Degrees of freedom (df) used was the difference between the two models.

The levels of significance per line for model 2 and any significant alleles in model 3 are given in the table below.

Table 4

		TNB		NBA	
Line	Model /allele	Signif-icance	Allele substit ution effect	Signif-icance	Allele substit ution effect
7	2	P<0.10		P<0.10	
	3/OPN122	P<0.05	1.30	P<0.05	-1.37
93	2	P<0.10		P<0.05	
	3/OPN112	P<0.10	-0.87	P<0.10	-0.92
	3/OPN132	NS	+0.49	P<0.05	+0.72
	3/OPN154	P<0.10	-0.72	NS	-0.46
94	2	P<0.01		P<0.05	
	3/OPN124	P<0.10	-0.83	P<0.10	-0.74
	3/OPN132	P<0.01	+1.62	P<0.05	+1.42

The following conclusions can be drawn from this data:

1. OPN accounted for a significant amount of variation in litter size (after including ESR) for L07 (P<0.10); L93 (TNB: P<0.10; NBA: P<0.05) and L94 (TNB: P<0.01; NBA: P<0.05).

2. OPN allele 132 showed a significant positive effect on litter size in L93 and L94.

3. Other alleles OPN122 (L07), OPN112 and OPN154 (L93) and OPN124 (L94) showed significant negative effects.

EXAMPLE 3

Genomic DNA samples from a further line L03 (another large white based line) were obtained and analysed. The results are shown below in table 5. 416 animals with 1,010 litter records were analysed.

Several different models were run. All models included the effect of farm-month farrowed, parity and sire. ESR was fitted as a co-variate in all analyses.

Traits analysed were total number born (TNB) and number born alive (NBA).

Models used:

1. Total number born= farrow-month+sire+ESR+OPN allele
2. TNB or NBA= farrow-month+sire+ESR+OPN112+OPN122 etc

Table 5

		TNB		NBA	
Line	Model/Allele	Significance	Allele substitution effect	Significance	Allele substitution effect
03	1/OPN124	P<0.01	+0.72		
	1/OPN136	P<0.15	-0.27		
	1/OPN138	P<0.15	+2.04		
	1/OPN142	P<0.15	-0.27		
	2/OPN112	NS	+0.34	NS	+0.58
	2/OPN122	NS	-o.13	NS	-0.18
	2/OPN124	<0.05	+0.65	NS	+0.33
	2/OPN132	NS	+0.31	NS	+0.08
	2/OPN136	NS	-0.29	NS	-0.37
	2/OPN138	<0.15	+2.06	NS	+1.81
	2/OPN140	NS	-0.11	NS	-0.16
	2/OPN142	NS	-0.22	<0.15	-0.40
	2/OPN144	NS	-1.06	NS	-1.16
	2/OPN146	NS	+1.08	NS	+0.15
	2/OPN154	NS	+0.02	NS	-0.02

This data indicates that OPN 124 shows a significant (P<0.01) positive effect for TNB of 0.7 for each copy of the allele. In addition, OPN 142 showed a trend toward a negative effect on litter size in L03, a similar effect to that seen for L93.

As discussed above, another gene ESR, has been shown to affect litter size in pigs and it is likely that other

genes linked with litter size will be identified in the future. We investigated whether certain beneficial allele combinations of the two separate genes, OPN and ESR,provide an additive effect on litter size.

To test this possibility we looked at the association between litter size and various combinations of ESR and OPN alleles. The results presented below in tables 4 and 5 show that indeed beneficial alleles of OPN can combine positively with beneficial alleles of ESR, such that an even greater litter size advantage can be realised than can be achieved through using beneficial alleles of OPN or ESR alone.

Table 4: Allele substitution effect for OPN and ESR markers on litter size (TNB) in line 93 (L93)

Marker	**Allele substitution effect for TNB**	**Significance**
OPN 132	+0.49	ns
ESR B	+0.34	ns
OPN 132 or ESR B	+0.39	P<0.1

Table 5: Expected litter size (TNB) advantage for various combinations of OPN and ESR markers in line 93 (L93) based on data presented in Table 4

Genotype				Litter size effect (TNB)
ESR	ESR	OPN	OPN	
-	-	-	-	0.00
B	-	-	-	+0.34
B	B	-	-	+0.68
-	-	132	-	+0.49
-	-	132	132	+0.98
B	-	132	-	+0.83 (+0.78)
B	B	132	-	+1.17 (+1.12)
B	-	132	132	+1.32 (+1.27)
B	B	132	132	+1.66 (+1.56)

Litter size effects assume complete additivity (OPN 132= +0.49; ESR B= +0.34) exceptthose in brackets which assume the effect of OPN 132 or ESR B= +0.39.

CLAIMS

1. A method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:

(i) obtaining a sample of genomic DNA from a pig; and

(ii) analysing the genomic DNA obtained in (i) to determine which OPN allele(s) is/are present.

2. A method as claimed in claim 1 wherein the determination of OPN alleles in step (ii) comprises determining the presence of at least one allele associated with at least one DNA marker linked either directly or indirectly to OPN.

3. A method as claimed in claim 2 wherein the DNA marker is a microsatellite.

4. A method as claimed in claim 3 wherein the DNA marker is Sw1085, Sw194, Sw16, Sw790, So178 or Sw61.

5. A method as claimed in claim 4 wherein one or more primers capable of hybridising to a region associated with the microsatellite are added to the sample of genomic DNA followed by one or more cycles of PCR to generate primer extension products.

6. A method as claimed in claim 5 wherein the OPN allele or alleles present in the sample of genomic DNA is

determined by reference to the length of the primer extension product(s).

7. A method as claimed in claim 5 or claim 6 wherein one or more of the following primers are employed:

GCTAGTTAATGACATTGTACATAA;
CCAATCCTATTCACGAAAAAGC;
GTGTCATGAGGTTTTTTCCACTGC; or
CAACCCACTTGCTCCCAC.

8. A method of screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:

(i) obtaining a sample of genomic DNA from a pig;

(ii) hybridising the genomic DNA from (i) with one or more suitable primers;

(iii) carrying out one or more PCR cycles using the hybridised nucleic acid from (ii); and

(iv) analysing the length of the PCR product obtained in (iii).

9. A method as claimed in claim 8 modified by any one or more of the features of claims 2 to 4.

10. A method as claimed in claim 9 wherein one or more of the following primers are employed:

GCTAGTTAATGACATTGTACATAA;
CCAATCCTATTCACGAAAAAGC;
GTGTCATGAGGTTTTTTCCACTGC; or
CAACCCACTTGCTCCCAC.

11. A kit for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which comprises one or more reagents or materials capable of identifying OPN alleles in a sample of pig genomic DNA.

12. A kit as claimed in claim 11 which comprises reagents or materials capable of identifying alleles associated with DNA markers linked to the OPN gene.

13. A kit as claimed in claim 12 wherein the DNA marker is a microsatellite and the kit comprises one or more DNA primers capable of hybridising to a region of the genomic DNA associated with the microsatellite.

14. A kit as claimed in claim 13 wherein one or more of the following primers are included:

GCTAGTTAATGACATTGTACATAA;
CCAATCCTATTCACGAAAAAGC;
GTGTCATGAGGTTTTTTCCACTGC; or
CAACCCACTTGCTCCCAC.

15. A kit as claimed in claim 13 or claim 14 which includes standard PCR reagents.

16. A method of determining which allele or alleles for a DNA marker associated with the pig OPN gene is/are associated with larger litter size, which comprises the

steps of:

(i) obtaining genomic DNA from one or more pigs;

(ii) determining which allele or alleles are present for a particular DNA marker associated with the OPN gene;

(iii) comparing the result of step (ii) with a similar determination carried out for one or more pigs known to produce larger litter sizes.

17. A method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:

(i) obtaining a sample of genomic DNA from a pig;

(ii) analysing the genomic DNA obtained in step (i) to determine which OPN allele(s) is/are present; and

(iii) analysing the genomic DNA obtained in step (i) to determine which alele(s) of at least one other gene linked to litter size in pigs is/are present.

18. A method as claimed in claim 17 wherein the at least one other gene is the ESR gene.

19. A method as claimed in claim 17 or claim 18 modified by any one or more of the features of any one of

claims 2 to 7.

20. A kit for screening pigs to dtermine those more likely to produce larger litters, and/or those less likely to produce larger litters, which comprises one or more reagents or materials capable of identifying OPN alleles in a sample of pig genomic DNA, together with one or more reagents or materials capable of identifying alleles of at least one other gene linked to litter size in pigs in a sample of pig genomic DNA.

21. A kit as claimed in claim 20 wherein the at least one other gene is the ESR gene.

22. A kit as claimed in cliam 20 or claim 21 modified by one or more of the features of any one of claims 12 to 15.

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04 C12P19/34

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 18651 (IOWA STATE UNIVERSITY RESEARCH) 29 October 1992 cited in the application see the whole document ---	18-22
A	SCIENCE , vol. 263, 25 March 1994, pages 1771-4, XP002018359 ANDERSSON, L., ET AL.: "Genetic mapping of quantitative trait loci for growth and fatness." -----	

☐ Further documents are listed in the continuation of box C.

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9218651	29-10-92	AU-B-	660994	13-07-95
		AU-A-	1917692	17-11-92
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		CA-A-	2108579	20-10-92
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		JP-T-	6506837	04-08-94
		US-A-	5550024	27-08-96
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(54) Title: GENETIC MARKERS FOR PIG LITTER SIZE

(57) Abstract

Disclosed herein are genetic markers for pig litter size, methods for identifying such markers, and methods of screening pigs to determine those more likely to produce larger litters. The markers are based upon the presence or absence of polymorphisms in the pig estrogen receptor gene. Preferably, the polymorphism is a restriction fragment length polymorphism (RFLP). A 4.3 kilobase fragment obtained by digesting pig genomic DNA with the restriction endonuclease Pvu II and detecting the fragments with a probe comprising a detectably labeled human estrogen receptor gene is associated with increased litter size.

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GENETIC MARKERS FOR PIG LITTER SIZE

FIELD OF THE INVENTION

This invention relates generally to the detection of genetic differences for reproductive efficiency among pigs and particularly to genetic markers useful for identifying pigs more likely to produce larger litter sizes.

BACKGROUND OF THE INVENTION

Reproductive efficiency, which can be defined as the number of pigs produced per breeding female, is the major limiting factor in the efficient production of pork. The number of pigs born alive in the United States averages approximately 9.5 pigs per litter. Heritability for litter size is low (10%-15%), and standard genetic methods of selecting breeding females on the basis of past litter size have not been effective. Therefore, there is a need for an approach that deals with selection for reproduction at the cellular or DNA level.

Chinese breeds are known for reaching puberty at an early age and for their large litter size. American breeds are known for their greater growth rates and leanness. Thus, it would be desirable to combine the best characteristics of both types of breeds, thereby improving the efficiency of U.S. pork production. These efforts would be greatly assisted by the discovery of genes or genetic markers that are associated with increased litter size in pigs.

Reproduction in mammals takes place in response to a chain of events that occur between the brain and the reproductive organs. The steroid hormones, such as estrogen, play a crucial role. Steroid hormones interact with cells and tissues, initiating a series of events that result in the ability to reproduce successfully.

In pigs, estrogen, which is produced mainly by the ovaries, has profound effects on the uterus, brain, and pituitary gland. Estrogens modulate the onset of puberty, reproductive behaviors, cyclic release of gonadotropins, and feeding behavior. The effects of estrogens take place as a result of the binding of estrogen to specific receptor proteins found in the nucleus of the estrogen-responsive cells. McEwen, et al., Recent Prog. Horm. Res., 38:41-92 (1982).

The gene responsible for coding for the human estrogen receptor has been identified, and it is publicly available from the American Type Culture Collection. See ATCC Catalog Sept. 1990, page 112, entry 57681. The probe name pOR3 and is 1.3 kb. Green et al., Nature (London) 320:134-139 (1986), incorporated herein by reference. The human gene is known to be polymorphic as a result of restriction fragment length polymorphism (RFLP) analysis. Castagnoli et al., Nucl. Acids Res., 15:886 (1987); Coleman et al., Nucl. Acids Res., 16:7208 (1988). The functional differences relating to these different genotypes are not well understood, but they have been implicated in increased spontaneous abortions in humans with breast cancer. Lehrer et al., The Lancet, 335:622-624 (March 17, 1990).

The estrogen receptor gene has been isolated and sequenced for other species, but not for pigs. Koike et al., Nucl. Acids Res., 15:2499-2513 (1987) reports the isolation and sequencing of a cDNA clone of the rat uterus estrogen receptor. The authors state that a comparison of rat, human, and chicken estrogen receptor sequences indicates the presence of three highly conserved regions, suggesting that these regions play important roles in estrogen receptor function.

In addition, Koike et al. Biochemistry 26:2563-2568 (1987) reports the partial characterization of the porcine estrogen receptor binding site. The paper reports a fragment of about 30 kDa that probably corresponds to the hydrophobic C-terminal-half region and has a greater than 90% homology with the corresponding rat, human, and chicken sequences.

RFLP analysis has been used by several groups to study pig DNA. Jung et al., Theor. Appl. Genet., 77:271-274 (1989) discloses the use of RFLP techniques to show genetic variability between two pig breeds. Polymorphism was demonstrated for swine leucocyte antigen (SLA) Class I genes in these breeds. Hoganson et al., Abstract for Annual Meeting of Midwestern Section of the American Society of Animal Science, March 26-28, 1990 reports on the polymorphism of swine major histocompatibility complex (MHC) genes for Chinese pigs, also demonstrated by RFLP analysis. Jung et al. Animal Genetics, 20:79-91 (1989) reports on RFLP analysis of SLA Class I genes in certain boars. The authors state that the results suggest that there may be an association between swine SLA/MHC Class I genes and production and performance traits. They further

state that the use of SLA Class I restriction fragments, as genetic markers, may have potential in the future for improving pig performance.

Prior to the present invention, RFLP analysis has not been applied to the pig estrogen receptor gene, which has not even been identified. The present invention overcomes these deficiencies. It provides genetic markers, based upon the discovery of polymorphism in the pig estrogen receptor gene, which relate to increased litter size in pigs. This will permit the screening and genetic typing of pigs for their estrogen receptor genes. It will also permit the identification of individual males and females that would be expected to produce a litter size larger than the average for their breed.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a method of screening pigs to determine those more likely to produce larger litters.

Another object of the invention is to provide a method for identifying genetic markers for pig litter size.

A further object of the invention is to provide genetic markers for pig litter size.

Yet another object of the invention is to provide a kit for evaluating a sample of pig DNA.

Additional objects and advantages of the invention will be set forth in part in the description that follows,

and in part will be obvious from the description, or may be learned by the practice of the invention. The objects and advantages of the invention will be attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purpose of the invention, as embodied and broadly described herein, the present invention provides a method for screening pigs to determine those more likely to produce a larger litter when bred. A sample of genomic DNA is obtained from a pig, and the presence or absence of a polymorphism in the estrogen receptor gene correlated with increased litter size is determined. Preferably, the polymorphism is a restriction fragment length polymorphism.

The presence or absence of a specific fragment or RFLP pattern is identified by the following steps. First, the genomic DNA is digested with a restriction endonuclease that cleaves the pig estrogen receptor gene in at least one place. Second, the fragments obtained from the digestion are separated, preferably by gel electrophoresis. Third, the fragments are detected with a probe capable of hybridizing to them. This generates a restriction pattern. Finally, the restriction pattern is compared to a known RFLP pattern for this gene that is correlated with increased litter size. The second pattern is one obtained by using the same restriction endonuclease and the same probe or an equivalent probe. Preferably, the probe is the human estrogen receptor gene.

In another embodiment, the invention comprises a method for identifying a genetic marker for pig litter

size. Male and female pigs of the same breed or breed cross or similar genetic lineage are bred, and the number of offspring produced by each female pig is determined. The polymorphism in the estrogen receptor gene of each pig is determined and associated with the number of offspring. Preferably, RFLP analysis is used to determine the polymorphism, and most preferably, the genomic DNA is digested with the restriction endonuclease Pvu II. For pigs of the Meishan breed, such analysis produces a 4.3 kilobase fragment associated with increased litter size.

The invention further comprises a kit for evaluating a sample of pig DNA. At a minimum, the kit is a container with one or more reagents that identify polymorphism in the pig estrogen receptor gene. Preferably, the reagent is a probe that hybridizes with the pig estrogen receptor gene or fragments thereof. Preferably, the probe is the human estrogen receptor gene. Preferably, the kit further contains a restriction enzyme that cleaves the pig estrogen receptor gene in at least one place.

The accompanying figure, which is incorporated in and constitutes a part of this specification, illustrates one embodiment of the invention and, together with the description, serves to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows RFLP analysis of Duroc (lane 1) and Chinese (lanes 2-16) pig DNA using the human estrogen receptor gene probe.

DETAILED DESCRIPTION OF THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain the principles of the invention.

The invention relates to genetic markers for litter size in pigs. It provides a method of screening pigs to determine those more likely to produce a larger litter when bred by identifying the presence or absence of a polymorphism in the estrogen receptor gene that is correlated with increased litter size. As used herein, the term "increased litter size" means a significant increase in litter size above the mean of a given population.

The use of RFLPs is the preferred method of detecting the polymorphism. However, since the use of RFLP analysis depends ultimately on polymorphisms and DNA restriction sites along the nucleic acid molecule, other methods of detecting the polymorphism can also be used. Such methods include ones that analyze the polymorphic gene product and detect polymorphisms by detecting the resulting differences in the gene product.

RFLP analysis in general is a technique well-known to those skilled in the art. See, for example, U.S. Patents 4,582,788 issued April 15, 1986 to Erlich and 4,666,828 issued May 19, 1987 to Gusella, both of which are incorporated herein by reference. Broadly speaking, the technique involves obtaining the DNA to be studied, digesting the DNA with restriction endonucleases,

separating the resulting fragments, and detecting the fragments.

In the present invention, a sample of genomic DNA is obtained from a pig. Generally, peripheral blood cells are used as the source of the DNA. A sufficient amount of cells are obtained to provide a sufficient amount of DNA for analysis. This amount will be known or readily determinable by those skilled in the art. The DNA is isolated from the blood cells by techniques known to those skilled in the art.

In certain instances, it may be desirable to amplify the amount of DNA through the use of standard techniques, such as the polymerase chain reaction. This technique is described in U.S. Patents 4,683,195, issued July 28, 1987 to Mullis et al., 4,683,202, issued July 28, 1987 to Mullis, 4,800,159 issued January 24, 1989 to Mullis, et al., 4,889,818 issued December 26, 1989 to Gelfand, et al., and 4,902,624, issued February 20, 1990 to Columbus, et al., all of which are incorporated herein by reference.

The isolated DNA is then digested with a restriction endonuclease that cleaves or cuts DNA hydrolytically at a specific nucleotide sequence, called a restriction site. Such endonucleases, also called restriction enzymes, are well-known to those skilled in the art. For the present invention, one should be chosen that cleaves the pig estrogen receptor gene in at least one place, producing at least two fragments of the gene. A determination is made as to whether or not such fragments are polymorphic and if the polymorphism is associated with

litter size by techniques known in the art in conjunction with the teachings contained herein. Preferably, such restriction endonuclease is Pvu II. The amount of such enzyme to be added to the sample containing the pig DNA and the other appropriate conditions for treating the sample will be readily determinable to persons skilled in the art, given the teachings contained herein.

The restriction fragments are then analyzed by known techniques that generally involve either the separation of the fragments to obtain a particular pattern or the determination of different sizes of the fragments. The preferred technique for doing so is gel electrophoresis.

In this technique, the digested fragments are separated in a supporting medium by size under the influence of an applied electric field. Gel sheets or slabs, such as agarose or agarose-acrylamide, are typically used as the supporting medium. The sample, which contains the restriction fragments, is added to one end of the gel. One or more size markers are run on the same gel as controls to permit an estimation of the size of the restriction fragments. This procedure generally permits a degree of resolution that separates fragments that differ in size from one another by as little as 100 base pairs.

The separated fragments preferably are then denatured and transferred physically from the gel onto a filter, preferably a nylon membrane, by contacting the gel with the filter in the presence of appropriate reagents and under appropriate conditions that promote the transfer of the DNA. Such reagents and conditions are well-known to

those skilled in the art. Thus, the relative positions of the DNA fragments resulting from the separation procedure are maintained.

The next step involves the detection of the various categories of sizes of the fragments or, alternatively, the detection of a fragment of a particular size. The latter may be of particular interest because it is a genetic marker associated with increased litter size. In either case, the preferred technique is the use of a hybridization probe. Such a probe is an oligonucleotide or polynucleotide that is sufficiently complimentary or homologous to the fragments to hybridize with them, forming probe-fragment complexes. Preferably, the probe is a cDNA probe. The oligonucleotide or polynucleotide is labeled with a detectable entity. This permits the detection of the restriction fragments, to which the probes are hybridized. The probes are labeled by standard labeling techniques, such as with a radiolabel, enzyme label, fluorescent label, biotin-avidin label, and the like.

In the present invention, a cDNA for the human estrogen receptor gene is used as the polynucleotide of the probe. Preferably, the detectable moiety is ^{32}P or biotin-avidin. The inventors have discovered that this probe is sufficiently homologous to the pig estrogen receptor gene to bind to it and to the various fragments produced by restriction endonucleases. However, other substantially equivalent probes can be determined by those skilled in the art, given the teachings contained herein. As used herein, a probe that is "substantially equivalent" to the human estrogen receptor gene probe is one that hybridizes to the same polymorphic fragments of digests of the pig estrogen

receptor gene as does the human estrogen receptor gene probe when the same restriction enzyme is used. For example, particular fragments that are associated with pig litter size can be sequenced by known techniques, and synthetic probes can be prepared, also by known techniques.

In the preferred method, the probes are contacted with the nylon membrane that contains the restriction fragments for a sufficient period of time and under appropriate hybridizing conditions for the probes to hybridize to the fragments. The filter is then preferably washed to remove unbound probes and other unwanted materials.

The probe-fragment complexes, which are bound to the filter, are then detected by known techniques. For example, if the probe has been radioactively labeled (^{32}P), detection involves contacting the nylon membrane paper with a piece of radiosensitive film. Following an appropriate exposure period, the fragments of interest, including control fragments, are visualized.

The detection step provides a pattern, resulting from the separation of the fragments by size. Comparison of these fragments with control fragments of known size that have also been run on the same gel permits the estimation of the size of the various groups of fragments. The various polymorphisms in the pig estrogen receptor gene are then determined by comparison of the patterns produced by similar analysis of DNA from a number of different pigs. For some of the individual pigs, the patterns will differ from the usual pattern produced by most of the other pigs. This will be due to one or more restriction fragment length

polymorphisms, i.e., restriction fragments of a different length produced by the endonuclease that cuts the pig estrogen receptor gene. This indicates different base pair sequences in such pigs.

Once a particular RFLP has been identified, i.e., a restriction fragment of a particular length, a probe to this fragment may be constructed by the use of known techniques. This permits alternative and faster formats for detecting such polymorphism. For example, once the DNA is digested, a sandwich hybridization format can be used. Such an assay is disclosed in U.S. Patents 4,486,539 issued December 4, 1984 to Ranki, et al., and 4,536,419 issued January 7, 1986 to Ranki, et al., both of which are incorporated herein by reference. The sample is brought into contact with a capture probe that is immobilized on a solid carrier. The probe binds the fragment. The carrier is then washed, and a labeled detection probe is added. After additional washing, the detection probe is detected, thereby demonstrating the presence of the desired fragment.

Once the RFLP pattern has been determined or a particular polymorphic fragment has been determined, it is compared to a second, known RFLP pattern or fragment that is correlated with increased litter size. This second pattern or fragment has also been determined from the pig estrogen receptor gene, using the same restriction endonuclease as the first and the same probe or an equivalent thereof.

In an alternative embodiment of the invention, the restriction fragments can be detected by solution hybridization. In this technique, the fragments are first

hybridized with the probe and then separated. The separated probe-fragment complexes are then detected by detecting the detectable moiety in the probe as discussed above. Generally, such complexes are detected on the gel without transfer to filter paper.

Although the above methods are described in terms of the use of a single restriction enzyme and a single probe, the methods are not so limited. One or more additional restriction enzymes and/or probes can be used, if desired. Additional enzymes, breeds, and constructed probes can be determined through routine experimentation.

Genetic markers for pig litter size are determined as follows. Male and female pigs of the same breed or breed cross or derived from similar genetic lineages are mated. The number of offspring produced by each female pig is determined. RFLP analysis of the parental DNA is conducted as discussed above in order to determine polymorphisms in the estrogen receptor gene of each pig. The polymorphisms are associated with the number of offspring. At least 20 and preferably at least 40 female pigs are used in making these determinations. The number of times each female produces a litter (i.e., the parity) is at least 1 time. Preferably, the cycle of breeding and giving birth is repeated at least 2 times and most preferably 3 times. The preferred breeds of pigs are Meishan, Fengjing, Minzhu, Duroc, Hampshire, Landrace, Large White, Yorkshire, Spotted Poland China, Berkshire, Poland China, and Chester White. The most preferred breeds are Duroc, Hampshire, Landrace, Large White, Yorkshire, and Chester White. When this analysis is conducted for the Meishan breed and the polymorphism is determined by RFLP analysis using the

restriction endonuclease Pvu II, a 4.3 kilobase fragment is associated with increased litter size.

The reagents suitable for applying the methods of the invention may be packaged into convenient kits. The kits provide the necessary materials, packaged into suitable containers. Preferably, the containers are also supports useful in performing the assay. At a minimum, the kit contains a reagent that identifies a polymorphism in the pig estrogen receptor gene that is associated with an increased litter size. Preferably, the reagent is a probe that hybridizes with the pig estrogen receptor gene or fragments thereof. Preferably, both the probe and a restriction enzyme that cleaves the pig estrogen receptor gene in at least one place are included in the kit. In a particularly preferred embodiment of the invention, the probe comprises the human estrogen receptor gene, a pig estrogen receptor gene, or a gene fragment that has been labelled with a detectable entity and the restriction enzyme comprises Pvu II. Preferably, the kit further comprises additional means, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, etc. may also be included, if desired.

The methods and materials of the invention may also be used more generally to evaluate pig DNA, genetically type individual pigs, and detect genetic differences in pigs. In particular, a sample of pig genomic DNA may be evaluated by reference to one or more controls. RFLP analysis is performed with respect to the pig estrogen receptor gene, and the results are compared with a control. The control is the results of a RFLP analysis of the pig

estrogen receptor gene of a different pig. Similarly, a pig may be genetically typed by obtaining a sample of its genomic DNA, conducting RFLP analysis of the estrogen receptor gene in the DNA, and comparing the results with a control. Again, the control is the results of RFLP analysis of the estrogen receptor gene of a different pig. Finally, genetic differences among pigs can be detected by obtaining samples of the genomic DNA from at least two pigs, identifying the presence or absence of polymorphism in the estrogen receptor gene, and comparing the results.

These assays are useful for identifying genetic markers relating to litter size, as discussed above, for identifying other polymorphisms in the estrogen receptor gene that may be correlated with other characteristics, and for the general scientific analysis of pig genotypes and phenotypes.

It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. The examples of the products and processes of the present invention appear in the following examples.

EXAMPLE 1

Genetic Marker for Increased Litter Size in Meishan Pigs

Materials and Methods

The procedures for detecting the restriction fragment length polymorphisms (RFLPs) were as follows. Ten ml of sterile blood were obtained from each pig. Isolation of genomic DNA was then performed from white blood cells, followed by digestion by Pvu II restriction endonuclease, Southern blotting, and hybridization with the estrogen receptor gene probe as outlined in Flanagan et al., Immunogenetics 27:465-469 (1988), incorporated herein by reference. Molecular sizes of the restriction fragments were determined by comparison with molecular size markers for Hind III cut lambda DNA restriction fragments run in parallel on the hybridization gels. The estrogen receptor probe was a 1.3 kb probe from the estrogen receptor gene isolated from humans (locus ESR) that was obtained from The American Tissue Culture Collection NIH repository of Human and Mouse DNA Probes (ATCC No. 57681) and labeled with ^{32}P.

Results

Using the human estrogen receptor gene as a probe, we have used RFLP analysis on Chinese, American, and NIH miniature pigs to detect genetic differences for the homologous estrogen receptor locus in the pig. Our results reveal that there are at least four fragments that are polymorphic in the pig. These fragments are at 3.7, 4.3, 5.0, and 7.7 kb.

Further, we investigated whether the polymorphic restriction fragment patterns were related to litter size in our original 22 Meishan females. See Table 1. Based on our results, having the 4.3 kb fragment seems to increase the litter size, while not having the 4.3 kb seems to be a disadvantage. These data indicate that we have found a gene marker for litter size in Meishan pigs.

Table 1. Means and standard errors of litter size in Meishan females by parity and estrogen receptor fragment.

		Parity		
Fragments		1	2	3
With 4.3 kb	NB	12.7 ± .84	14.2 ± 1.16	16.3 ± .33
	NBA	12.4 ± .81	12.8 ± .92	15.0 ± 1.53
	N	7	5	3
Without 4.3 kb	NB	11.4 ± .71	11.4 ± 1.31	13.5 ± 1.84
	NBA	10.9 ± .65	10.2 ± 1.17	13.3 ± 1.79
	N	14	11	4

NB = Number Born, NBA = Number Born Alive, N = Number of litters

WE CLAIM:

1. A method of screening pigs to determine those more likely to produce larger litters comprising the steps of:

obtaining a sample of genomic DNA from a pig; and

determining the presence or absence of a polymorphism in the estrogen receptor gene associated with increased litter size.

2. The method of claim 1 wherein said polymorphism is a restriction fragment length polymorphism (RFLP).

3. The method of claim 2 wherein said step of identifying the presence or absence of said RFLP comprises the steps of:

digesting said genomic DNA with a restriction endonuclease that cleaves the pig estrogen receptor gene in at least one place;

separating the fragments obtained from said digestion;

detecting said fragments with a probe capable of hybridizing to said fragments, thereby generating a restriction pattern; and

comparing said pattern with a second RFLP pattern for the pig estrogen receptor gene obtained by using said

restriction endonuclease and said probe or equivalent thereof, wherein said second RFLP pattern is associated with increased litter size.

4. The method of claim 3 wherein said restriction endonuclease is Pvu II.

5. The method of claim 3 wherein said separation is by gel electrophoresis.

6. The method of claim 3 wherein said probe is the human estrogen receptor gene or a substantial equivalent thereof, said gene or substantial equivalent being labeled with a detectable entity.

7. The method of claim 3 wherein said step of comparing said restriction patterns comprises identifying specific fragments by size and comparing the sizes of said fragments.

8. The method of claim 3 further comprising the step of amplifying the amount of said pig estrogen receptor gene prior to said digestion step.

9. The method of claim 2 wherein said step of identifying the presence or absence of said RFLP comprises the steps of:

adding to said sample a restriction enzyme that cleaves the pig estrogen receptor gene into fragments; and

detecting the different sizes of said fragments.

10. The method of claim 9 wherein said step of detecting the different sizes of said fragments comprises the steps of:

separating said fragments by size using gel electrophoresis in the presence of a control DNA fragment of known size;

contacting said separated fragments with a probe that hybridizes with said fragments to form probe-fragment complexes; and

determining the size of the separated fragments by detecting the presence of the probe-fragment complexes and determining their relative positions with respect to said control DNA fragment.

11. The method of claim 2 wherein said step of identifying the presence or absence of said RFLP comprises the steps of:

adding to said sample a restriction enzyme that cleaves the pig estrogen receptor gene into fragments; and

determining the presence or absence of a DNA fragment of known size.

12. The method of claim 11 wherein said step of determining the presence or absence of said DNA fragment of known size comprises the steps of:

contacting said sample with a probe that hybridizes with said DNA fragment of known size to form probe-fragment complexes; and

detecting the presence or absence of said complexes.

13. A method for identifying a genetic marker for pig litter size comprising the steps of:

breeding male and female pigs of the same breed or breed cross or derived from similar genetic lineages;

determining the number of offspring produced by each female pig;

determining the polymorphism in the estrogen receptor gene of each pig; and

associating said number of offspring with said polymorphism.

14. The method of claim 13 wherein said polymorphism is determined by RFLP analysis.

15. The method of claim 13 wherein said breed is selected from the group consisting of Meishan, Fengjing, Minzhu, Duroc, Hampshire, Landrace, Large White, Yorkshire, and Chester White.

16. The method of claim 15 wherein said breed is Meishan and said polymorphism is determined by RFLP analysis.

17. The method of claim 16 wherein said RFLP analysis comprises digestion of genomic DNA with the restriction endonuclease Pvu II.

18. The method of claim 17 wherein said RFLP analysis produces a 4.3 kilobase fragment correlated with increased litter size.

19. A method for evaluating a sample of pig genomic DNA by reference to a control comprising the steps of:

conducting a RFLP analysis of the pig estrogen receptor gene; and

comparing the results of said analysis with said control, wherein said control comprises the results of a RFLP analysis of the pig estrogen receptor gene of a different pig from the pig that provided the sample of genomic DNA.

20. A method of genetically typing a pig comprising the steps of:

obtaining a sample of genomic DNA from said pig;

conducting RFLP analysis of the estrogen receptor gene in said DNA; and

comparing the results of said analysis with a control, wherein said control comprises the results of a

RFLP analysis of the estrogen receptor gene of a different pig.

21. A method of detecting genetic differences among pigs comprising the steps of:

obtaining samples of genomic DNA from at least two pigs;

identifying the presence or absence of a polymorphism in the estrogen receptor gene in said samples; and

comparing the results of said identification step.

22. The method of claim 21 wherein said step of identifying the presence or absence of a polymorphism comprises conducting RFLP analysis of said estrogen receptor gene.

23. The genetic marker identified by the method of claim 13.

24. A genetic marker for increased litter size in pigs comprising the 4.3 kilobase restriction fragment obtained by digesting DNA isolated from said pigs with the restriction endonuclease Pvu II.

25. A kit for evaluating a sample of pig DNA comprising, in a container, a reagent that identifies polymorphism in the pig estrogen receptor gene.

26. The kit of claim 25 wherein said reagent is a probe that hybridizes with the pig estrogen receptor gene or fragments thereof.

27. The kit of claim 26 wherein said probe comprises the human estrogen receptor gene or equivalent thereof labeled with a detectable moiety.

28. The kit of claim 25 further comprising a restriction enzyme that cleaves the pig estrogen receptor gene in at least one place.

29. The kit of claim 28 further comprising means for detecting said probe.

30. The kit of claim 29 further comprising control means.

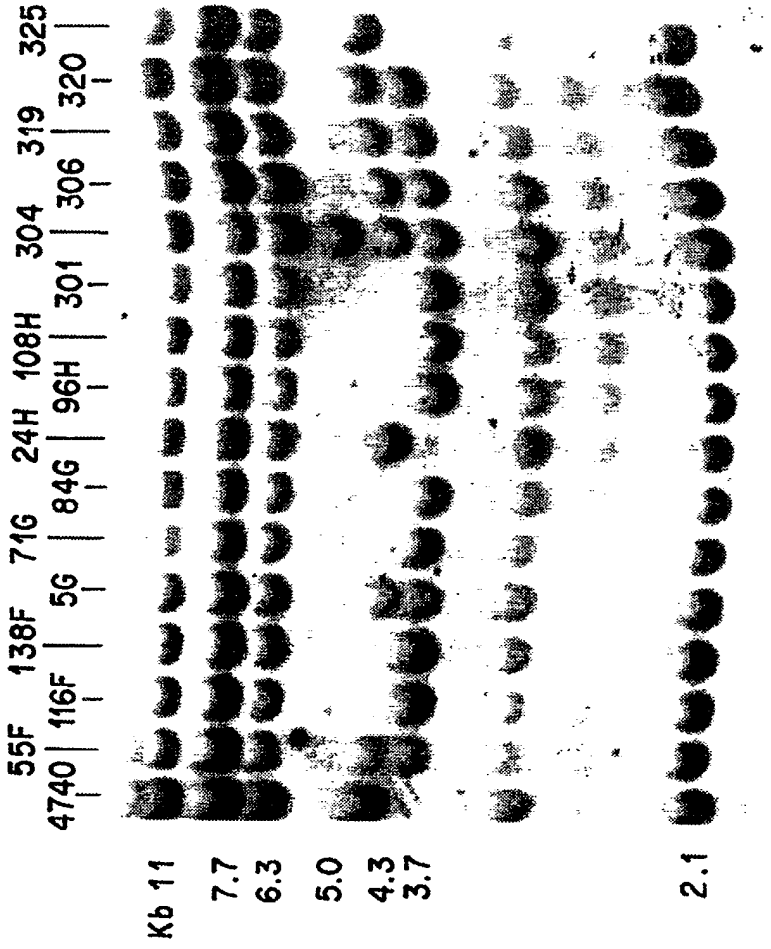
55F
4740
116F
138F
5G
71G
84G
24H
96H
108H
301
304
306
319
320
325
Kb 11
7.7
6.3
5.0
4.3
3.7
2.1

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03160

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :IPC (5): C12Q 1/68; C12P 19/34; C07H 15/12
US CL :U.S. CL.: 435/6, 91; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : U.S. CL.: 435/6, 91; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, DIALOG,
search terms: RFLP's, Pig, Swine, estrogen receptor.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nucleic Acids research, Vol. 16, No. 14, issued 1988, Coleman et al.,"Human estrogen receptor (ESR) gene locus: PssI dimorphism", page 7208, all document.	1-30
Y	Nucleic Acids Research, Vol. 15, No.2, issued 1987, Castagnoli et al.,"PvuII RFLP inside the human estrogen receptor gene" page 866, all document.	1-30
A	Biochemistry, Vol. 26, No.9, issued 1987, Koike et al.,"The steroid binding domain of porcine estrogen receptor", pages 2563-2568, all document.	1-30
A	Nucleic Acids Research, Vol. 15, No.6, issued 1987, Koike et al.,"Molecular cloning and characterization of rat estrogen receptor", pages 2499-2513, all document.	1-30
A	Animal Genetics, Vol. 20, issued 1989, Jung et al.,"Association of restriction fragment length polymorphisms of swine leucocyte antigen class I genes with production traits of Duroc and Hampshire boars", pages 79-91 all document.	1-30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 JULY 1992	Date of mailing of the international search report 31 JUL 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer MIGUEL ESCALLON PH.D. Telephone No. (703) 308-0376

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03160

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Immunogenetics, Vol 21, issued 1985, Chardon et al.,"Restriction fragment length polymorphism of the major histocompatibility complex of the pig", pages 161-171, all document.	1-30
A	Immunogenetics, Vol. 27, issued 1988, Flannagan et al.,"RFLP analysis of SLA class I genotypes in Duroc swine", pages 465-469, all document.	1-30
A	Theoretical Applied Genetics, Vol. 77, issued 1989, Jung et al.,"Genetic variability between two breeds based on restriction fragment length polymorphisms (RFLPs) of major histocompatibility complex class I genes in the pig", pages 271-274, all document.	1-30

UNITED STATES
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Commissioner for Patents
Washington, DC 20231
www.uspto.gov

DEC 20 2002

Dear Patent Business Customer:

The United States Patent and Trademark Office (USPTO) has begun testing the use of image technology to replace paper processing of patent applications. You have the opportunity to be involved with a historic prototype that is testing this electronic processing of patent applications within three art units (1634, 2827 and 2834) and the Office of Initial Patent Examination (OIPE).

What will be tested during the prototype?
In the prototype, the paper document contents of pending applications assigned to the three participating art units will be scanned into electronic image files. All processing and examination will be performed with the resulting electronic files during the prototype.

How will applicants be affected?
The attached information sheet details two process changes:

- Amendments should be submitted in a special format. Specifically, a new complete claim set with changes marked in strikeouts and underlining should be submitted. No clean copy of the amended claims is necessary. Please see the OG Notice of 12/17/02 (http://www.uspto.gov/web/offices/com/sol/og/2002/week51/patimag.htm.)
- US patent and US published application references will not be mailed with Office actions. These references are available free of change on our website.
- Applicants are requested to use eIDS for submission of any IDS. For assistance with eIDS, you may contact the Patent Electronic Business Center (toll free at (866) 217-9197).

What are the official files?
During the prototype, the Office will maintain in a central location the original papers that constitute the official file. Over the next few months, the Office will be taking steps to have the electronic file deemed the official file.

How long will the prototype last?
It is expected that the prototype program will incrementally migrate into a production system, with the goal of eliminating paper patent applications files by early summer.

Want more information or to provide feedback?
For more information on the prototype or to provide feedback, please contact :
Search and Information Resources Administration at image.processing@uspto.gov

Through your participation, USPTO will have an opportunity to test the electronic processing of patent applications and meet its goal of delivering high quality products through implementation of electronic patent processing by the end of 2004. Thank you for your support for this exciting activity and for helping the USPTO achieve its goal of providing better service through e-Government.

Sincerely,

Nicholas P. Godici

Nicholas P. Godici
Commissioner for Patents

NOTIFICATION OF REQUESTED NEW FORMAT FOR AMENDMENTS AND/OR RESPONSES FILED IN ART UNITS 1634, 2827, AND 2834

The United States Patent and Trademark Office (USPTO) is currently conducting a prototype of electronic application processing and examination in **Art Units 1634, 2827, and 2834**. To facilitate the prototype, the following changes in format for Amendments and/or Responses filed in those Art Units are requested.

Requested Format of Amendments and Responses during the prototype.

Each section of an Amendment and/or Response (e.g., Claim Amendments, Specification Amendments, Remarks) should begin on a separate sheet to facilitate separate indexing and scanning of the document. *For example*, in an Amendment containing a.) introductory comments, b.) amendments to the claims, c.) amendments to the specification, and d.) remarks, each of these sections should begin on a separate sheet. **For each amendment filed in Art Units 1634, 2827, and 2834, the requirement to provide two sets of claims (a clean version and a marked up version), as set forth in 37 CFR 1.121(c), will be waived where the following format is employed.**

Each amendment that includes a change to an existing claim, or submission of a new claim shall be made by submitting **a summary document with the status of all claims and the text of all pending claims as follows:**

(1) The status of all of the claims in the application, including any previously canceled or withdrawn claims, must be summarized in each amendment document. Status is indicated by a parenthetical expression following the claim number (e.g. (original), (currently amended), (previously amended), (canceled), (withdrawn), or (new)). The text of all pending claims must be submitted each time any claim is amended. Canceled and withdrawn claims may be indicated by only the claim number and status.

(2) All claims being currently amended must be submitted with markings to indicate the changes that have been made. The changes in any amended claim may be shown by strikethrough (for deleted matter) or underlining (for added matter), or by any equivalent marking system.

(3) The text of pending claims not being amended must be presented in each amendment document in clean version, i.e., without any markings. Any claim presented in clean version will constitute an assertion that it has not been changed relative to the immediate prior version.

(4) A claim may be canceled by merely providing an instruction to cancel. Any claims added by amendment must be indicated as (new).

(5) All of the claims in each amendment paper must be presented in ascending numerical order. Consecutive canceled or withdrawn claims may be aggregated into one statement (e.g. Claims 1 – 5 (canceled)).

Amendments to the specification are performed in the conventional manner (37 CFR 1.121(b)).

Patent Copies

Additionally, applicants and practitioners will no longer be required to provide copies of U.S. Patents and Published U.S. Patent Applications cited in any Information Disclosure Statement (IDS) submitted to the USPTO during the prototype and in applications assigned to the three art units. It is requested that eIDSs be used to file all IDS papers for applications before the prototype Art Units. Similarly, during the prototype, copies of U.S. Patents and Published U.S. Patent Applications cited by an examiner during prosecution of an application will not be provided to applicants in Office actions from these Art Units. These documents are available from the USPTO web site, www.uspto.gov for free download. Cited foreign patents and published applications and non-patent literature will be mailed by conventional processing.

The above requested new format and procedures are applicable during the prototype only to applications assigned to Art Units 1634, 2827, and 2834. Any questions regarding these requirements may be directed to image.processing@uspto.gov or one of the Supervisory Patent Examiners of these Art Units 1634 – Gary.Jones@uspto.gov; 2827 – Dave.Talbott@uspto.gov or 2834 – Nestor.Ramirez@uspto.gov .

Example:

Claims 1-5 (canceled) (Note: consecutive canceled or withdrawn claims may be aggregated)

Claim 6 (withdrawn)

Claim 7 (previously amended): A bucket with a handle.

Claim 8 (currently amended): A bucket with a ~~green~~ blue handle.

Claim 9 (withdrawn)

Claim 10 (original): A bucket with a wooden handle.

Claim 11: (new): A bucket with plastic sides and bottom.

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